CANCER RESEARCH

VOL. 11 JANUARY 1951 No. 1

CONTENTS

THE OFFICIAL ORGAN OF THE

AMERICAN ASSOCIATION FOR CANCER RESEARCH, INC.

Published by THE UNIVERSITY OF CHICAGO PRESS

CANCER RESEARCH

This journal is sponsored by The American Association for Cancer Research, Inc.; The Anna Fuller Fund; Cancer Research Division, Donner Foundation, Inc.; The Jane Coffin Childs Memorial Fund for Medical Research; The Elsa U. Pardee Foundation; and The American Cancer Society.

CANCER RESEARCH is published monthly for Cancer Research, Inc., by the University of Chicago Press, 5750 Ellis Avenue, Chicago 37, Illinois. Subscriptions are by volume only and are payable in advance. The subscription price is $12.50 per volume; the price of single issues is $1.25. Orders for less than a full volume will be charged at the single-issue rate. Postage is prepaid by the publishers on all orders from the United States and its possessions. No extra charge is made for countries in the Pan American Postal Union. Postage is charged extra as follows: For Canada and Newfoundland, 50 cents per volume (total $13.00), 5 cents per issue (total $1.30); for all other countries in the Postal Union, $1.00 per volume (total $13.50), 10 cents per issue (total $1.35).

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Entered as second-class matter, February 15, 1949, at the post office at Chicago, Ill., under the Act of March 3, 1879.

Acceptance for mailing at special rate of postage provided for in United States Postal Act of October 3, 1917, Section 1103, amended February 28, 1925, authorized June 1, 1950.

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VOLUME 11 JANUARY 1951 NUMBER 1

Cytochemical Studies of Mammalian Tissues: The Isolation of Cell Components by Differential Centrifugation: *A Review**

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Cytologists have appreciated for many years that the cell is not a homogeneous mass of protoplasm but that it contains several discrete structures that can be seen and identified in all cells of the plant and animal kingdom. The function and the chemical composition of these structures have been the source of considerable speculation and the inspiration for much experimental work, mainly of an indirect nature. In the present review, an attempt will be made to examine critically one of the methods that have been devised to determine directly the chemical composition and the function, in terms of enzymatic activity, of the structural components of the cell. This method involves the mechanical rupture of tissue cells in a suitable medium followed by the isolation of the cell components by means of differential centrifugation.

The method of differential centrifugation of broken cell suspensions was introduced in 1934 by Bensley and Hoerr (11), who described the isolation of mitochondria from guinea pig liver. Since the paper of Bensley and Hoerr, numerous publications have appeared dealing with the isolation of individual cellular components. Many have dealt with the isolation of nuclei (9, 34). Others have described improvements in the procedures for the isolation of mitochondria (19–21, 23–25, 60), while still others have dealt with the isolation of chromosomes (28, 90–92) and submicroscopic particles (19–21, 23–25, 60, 77), including glycogen particles (24, 76, 77) in a submicroscopic form. More recent papers have described procedures for the complete fractionation of a tissue into nuclei, mitochondria, submicroscopic particles, and soluble material (60, 122, 125, 129).

The fact that particulate components of the cell can be isolated from broken cell suspensions in almost unlimited yields makes it apparent that this technic is capable of utilizing to the fullest extent modern biochemical methods for the study of cell composition and function. In this respect, the procedure of cell fractionation is more versatile than either of two other methods at present available in the field of cytochemistry; namely, the histochemical technics (44) and the submicro technics of Linderstrom-Lang, Holter, and their associates (82). The former, aside from the possible artifacts introduced by the necessary procedures of freezing or fixation, are severely limited by their dependence on microscopic visualization for the localization of an enzymatic reaction or a chemical compound and as a result usually employ indirect methods of analysis which cannot readily be quantitated and generally entail the possibility of still further artifacts. The latter technic, which most nearly approaches the ideal cytochemical tool in that it is aimed at the study of single cells and portions thereof, is apparently not as yet sufficiently sensitive to be applied to

* For the sake of brevity and convenience the following abbreviations will be employed in this paper: DNA = desoxypentosenucleic acid; PNA = pentosenucleic acid; AMP = adenosine-5-phosphate; AMP-ase = AMP phosphatase; ATP = adenosinetriphosphate; ATP-ase = adenosinetriphosphatase; DPN = diphosphopyridine nucleotide; TPN = triphosphopyridine nucleotide; and DAB = 4-dimethylaminoazobenzene.

Received for publication October 19, 1950.

single mammalian cells. Although it is hoped that the results obtained with the cell fractionation technic will be checked by some other method, it will be extremely difficult for other methods to keep pace, owing to the versatility and wide applicability of the former. Accordingly, it is necessary to understand clearly the limitations of the cell fractionation technic and to define the criteria that identify an adequate isolation procedure.

Some investigators (14, 16, 33) have taken the view that the procedure of cell fractionation can be dismissed *a priori* on the grounds that the very act of cell rupture necessarily produces artifacts such as redistribution, adsorption, morphological alterations, etc. Although such artifacts are obvious problems, the dismissal of the procedure as an experimental tool, without concrete evidence of its uselessness and without the submission of an adequate alternative, constitutes a negative approach that is difficult to defend. The reviewers prefer to adhere to the *positive* approach of conducting cell fractionation experiments in such a way that possible artifacts may be tested experimentally.

In a consideration of artifacts likely to be encountered in the procedure of cell fractionation, it must first be realized that when the cell is disrupted, its structural components are released into distinctly abnormal surroundings. Since cytological studies have demonstrated that at least two cellular components, nuclei (141) and mitochondria (26, 32, 66, 95, 143, 144), possess well defined membranes, it appears entirely possible that these membranes may be so damaged in the process of cell disruption as to allow the escape of soluble substances. Although experiments providing direct evidence for the integrity of the membranes have not as yet been devised, strong indirect evidence can be offered by certain lines of approach. Thus, it seems likely that the isolation of a cell component, in morphological and cytologically unaltered form, will maintain the integrity of the membrane. In this respect, it has been shown (60) that the cytological and morphological properties of liver mitochondria are profoundly affected by the composition of the medium in which the liver cells are disintegrated. Only when the cells were disrupted in hypertonic solutions of nonelectrolytes was the morphology and cytology of the mitochondria maintained. Additional and more direct evidence for the integrity of the mitochondrial membranes has also been provided by the recent demonstration that the mitochondria contain large amounts of soluble proteins that can be released only by disruption of the mitochondrial membranes (57, 59). Another method that might be suggested to test the integrity of the membranes of particulate structures is that of equilibrating the isolated structure with a solution of the isotopically labeled material in question. Labeled material should appear within the structure if its membrane is permeable. A method peculiar to certain enzymes can also be used to test the integrity of particulate structures. Thus, in the case of cytochrome c it was found (128) that liver mitochondria isolated from water homogenates contained high concentrations of cytochrome c but that it was biologically inactive in the succinoxidase system present in the mitochondria. On the other hand, cytochrome c present in mitochondria isolated from isotonic saline or sucrose or hypertonic sucrose (128, 129) was highly active in the oxidation of succinate by the mitochondria.

A second important problem, namely, the possibility of adsorption of soluble material on cellular particles, can perhaps best be approached experimentally by washing the isolated particulate structure in various media. If the material is tightly bound to the structure and not adsorbed, repeated washing should fail to reduce either its total amount or its concentration. This has been shown to be the case for certain of the respiratory enzymes associated with liver mitochondria and microsomes (55, 56, 125). On the other hand, cytochrome c has been shown to be adsorbed on liver microsomes when these were isolated from water homogenates but not when the microsomes were isolated from saline or sucrose homogenates (128, 129). Furthermore, the cytochrome adsorbed on the microsomes was completely removed when the particles were washed with isotonic saline (128).

A consideration of the quantitative aspects of the intracellular distribution of the substance in question also has a decided bearing on the question of adsorption. Thus, if a large percentage of the substance present in the whole tissue can be shown to be localized in a single fraction, then the probability of adsorption would appear to be minimal. Some of the enzymes referred to in the preceding paragraph fulfill this condition. On the other hand, the finding of a *small* percentage of a substance in a fraction must be seriously considered as an adsorption phenomenon. In the case of isocitric dehydrogenase, for example, about 82 per cent of the enzyme activity was recovered in the soluble fraction and 12 per cent in the mitochondria (61). The significance of the latter seems rather doubtful and can probably be considered as the result of adsorption. Dounce (36) has argued that, for results to be significant, the proportion of the total enzyme in a particulate structure need not be

greater than the proportion of the total cell volume occupied by the particulate structure. Although this may be true, the actual proof that it is the case can come only from measurements on single cells.

The need for establishing balance sheets in which the summation of the activities of the tissue fractions is compared with that of the whole tissue has been emphasized repeatedly (56, 62, 122, 127). Such balance sheets serve several purposes, one of which has already been mentioned in the preceding paragraph. In addition, tabulations of this type test the validity of the analytical methods and of the assay procedures employed, and in the case of enzymes indicate whether inhibitors or activators are present. By judicious recombination of fractions it is possible to determine which fraction contains the inhibitor or activator and thus to learn how the activities of the various cell fractions are integrated to reproduce the activity of the cell as a whole.

METHODS FOR THE ISOLATION OF PARTICULATE CELL STRUCTURES

An adequate method for the preparation of a particulate component of the cell should provide that component in pure form, morphologically and cytologically unaltered, and in high yield. Because of the possibility of heterogeneity of cells and of cell fractions and because many of the present applications of the cell fractionation technic involve comparative studies on different tissues, the need for large yields is immediately obvious.

One of the factors affecting yield in cell fractionation studies is the method used for cell disruption. This was discussed in a previous publication (127), and it was concluded that the best available method involved the use of the Potter-Elvehjem homogenizer (107), because it produced maximum degrees of cell rupture without causing breakage of subcellular elements. In the experience of the reviewers, the Waring Blendor is entirely unsatisfactory for the preparation of homogenates suitable for cell fractionation studies. The subjection of liver, for example, to the action of the Waring Blendor for periods sufficient to disrupt most of the cells results in the breakage of many nuclei and apparently even of some mitochondria. Excessive frothing occurs, and the temperature is controlled with difficulty. A recent report (75) also indicates that the Waring Blendor is capable of inactivating some enzymes and related compounds.

Another factor affecting the yield is that of aggregation of particles. Thus, in the case of liver homogenates it was demonstrated that electrolytes caused pronounced aggregation of mitochondria and that this aggregation was largely prevented by the use of nonelectrolytes (60). More recently, it has been reported that agglutination of cytoplasmic particles in electrolyte solutions can be prevented by the use of heparin (63).

Another point to be considered is the tissue chosen for cell fractionation studies. The ideal tissue would contain only one cell type. In practice, however, very few tissues can even approach this ideal. Among those that might be mentioned are liver, epidermis, and certain tumors. It is to be hoped that the technics of tissue culture may some day provide cells of all types in amounts sufficient for fractionation studies.

Isolation of nuclei.—Several methods have been employed for the isolation of mammalian nuclei. The first method involves the use of citric acid, and, in the procedure of Dounce (34) for the isolation of liver nuclei, only enough citric acid is added to attain a pH of 6. The yield of nuclei with this method has not been established, but, judging from the description of the procedure, the yield is probably low. A photomicrograph of nuclei isolated by this procedure shows quite clearly that the nuclei are altered morphologically, as shown by the presence of precipitated chromatin as well as well as by deviations of some of the nuclei from a characteristic spherical form. Unaltered nuclei are completely homogeneous under the microscope and, in the case of liver, are generally spherical in shape (73, 118, 137). It should be mentioned that nuclei of liver homogenates in either isotonic or hypertonic sucrose fulfill these cytological criteria (60, 73, 101, 102, 118, 137). The isolation of nuclei from homogenates of spleen in isotonic sucrose containing citric acid (4) has been described, but photomicrographs of the isolated nuclei, by means of which the morphological properties might be judged, were not presented. The addition of citric acid was stated to be necessary to prevent the loss of nucleoprotein from the nuclei during the isolation. However, the experiment which was stated to prove that DNA was lost from nuclei in sucrose proved that DNA could be extracted with molar NaCl from nuclei in sucrose but not from nuclei in sucrose containing citric acid. The latter is at variance with the findings of Dounce (34, 35).

Another method, devised by Behrens (9), consisted of lyophilization of the tissue, grinding of the dry tissue to disrupt the cells, suspension of the powder in an organic solvent mixture of suitable density, and centrifugation of the resulting suspension at a suitable speed. Dounce and his associates (37) have recently applied this method to the study of amino acid distribution in nuclei and have published the first photomicrograph of such nuclei. These nuclei show morphological al-

terations similar to those obtained with citric acid. The adequacy of the Behrens' method for enzyme investigations might also be questioned, since it is well known that some enzyme systems are inactivated by freezing and drying. However, the method does possess the important advantage that artifacts produced by the redistribution of materials should be absent. From the latter standpoint, the Behrens' method may prove valuable for testing the results of fractionations conducted in aqueous solutions.

Another preparation that has proved to be of considerable use in comparative studies is the nuclear fraction obtained as a first step in the complete fractionation of tissues (60, 122). This fraction contains all the nuclei of the tissue, in addition to some unbroken cells and mitochondria. Despite these impurities, however, much useful information has been supplied by a study of this fraction. Barnum *et al.* (8) have recently subjected the nuclear fraction to further treatment with citric acid in order to obtain nuclei sufficiently pure for studies on uptake of radioactive phosphorus. Nuclei so treated appear to have lost a large amount of their original protein, however.

Recent cytological studies by Pollister and Leuchtenberger (105) have far-reaching implications in regard to methods for the isolation of nuclei and perhaps for the isolation of other particulate components of the cell as well. These authors have found that the ratio of protein to DNA in nuclei in tissue sections was several-fold greater than the ratios reported by various authors for nuclei isolated with citric acid. It is not clear at present, however, whether the cytologically determined ratios are correct, because of the difficulties involved in assigning absolute values to the extinction values determined on the nuclei in tissue sections (119, 137). However, these authors performed an experiment which would appear to be independent of the above objection. They incubated rat liver slices in isotonic saline prior to fixation and noted a marked loss of nonhistone-type protein from the nuclei. This experiment raises several important questions, e.g., whether protein is lost from nuclei at the moment of cell rupture or whether a medium can be devised to prevent loss of protein from nuclei. Recent experiments by Dounce *et al.* (37), with the technic of Behrens for the isolation of nuclei, appear to confirm the cytological results. Nuclei isolated by this method had much higher protein-DNA ratios than did those isolated in dilute citric acid (although the ratio in the latter instance was also much higher than that for nuclei isolated by citric acid technics at a lower pH than that employed by Dounce). However, the nuclei isolated by the Behrens' method were peculiar in that their *PNA* content was greater than their DNA content. If correct, this finding would mean that about one-third of the PNA present in liver is localized in the nucleus and would imply that isolation of nuclei in aqueous media results in loss of not only protein from nuclei but also considerable amounts of PNA. It should be possible to check the latter findings cytologically.

In regard to improved media for the isolation of nuclei, it might be mentioned that several lines of evidence indicate that hypertonic solutions of nonelectrolytes may be necessary. The fact that nuclei appear normal morphologically in such media has already been mentioned above. In addition, it should be noted that the isolation of liver mitochondria in a morphologically and cytologically unaltered form was accomplished in such solutions (60). Furthermore, Opie (100) has found that tissue slices take up water when immersed in isotonic solutions but not when immersed in hypertonic solutions. There is thus apparently an increasing body of evidence that the osmotic pressure inside the liver cell is considerably greater than in blood serum.

Isolation of chromosomes.—Chromatin threads were isolated independently by Claude and Potter (28) and by Mirsky and Pollister (90). Subsequently, Mirsky and Ris (91) presented evidence that the chromatin threads possessed the morphological characteristics of chromosomes.

The isolation of chromosomes is accomplished by means of differential centrifugation after rupture of nuclear membranes by one of the following methods: grinding with sand, prolonged mixing in a Waring Blendor, or passage through a colloid mill. The yield of chromosomes has not been reported, although this can be estimated from the amount of DNA recovered in the chromosomes as compared to the DNA content of the whole tissue. The purity of the chromosomes was tested by applying immunological tests for serum proteins as well as cytological tests for cytoplasmic proteins. According to Mirsky (89), these tests were essentially negative. Ris and Mirsky (118) also point out that isolated chromosomes are derived from resting nuclei, in which the chromosome structures cannot be seen. They consider the isolation of structures possessing the morphological characteristics of chromosomes as evidence for the preexistence within the nuclei of chromosomal structures, even though invisible in the living cell. However, the possibility that chromosomes are *not* present as discrete structures within resting nuclei but are formed from submicroscopic or soluble ma-

terial at the moment of nuclear rupture must also be considered, inasmuch as Ris and Mirsky (118) have demonstrated that the nuclei from which the chromosomes were isolated had been altered morphologically by the electrolyte medium in which they were suspended.[1]

In experiments with isolated chromosomes, it must also be remembered that the chromosome preparation contains nucleoli, some of which apparently stick to the chromosomes and cannot be removed (28, 91). It is not clear whether all nucleoli are so trapped or whether only a few are. In any event, any property ascribed to chromosome preparations must be considered in the light of nucleolar contamination.

In regard to the method of isolation, Mirsky (89) dismisses the isolation of nuclei as a desirable step preliminary to rupture of nuclear membranes because of the undesirable properties of nuclei prepared in citric acid. It would appear desirable, however, to wash the minced tissue as completely as possible or to isolate the washed nuclear fraction in order to remove as much cytoplasm as possible prior to the rupture of the nuclear membranes.

Isolation of mitochondria.[2]—The isolation of mitochondria was first reported by Bensley and Hoerr (11). The details of their procedure, however, were not reported until a few years ago (54, 77). Meanwhile, Claude (19, 20) described the isolation from various tissues of large granules, which were at first considered to be secretory granules (19, 20). Subsequently, however, Claude stated that the large granule preparations were either mitochondria or mixtures of mitochondria and secretory granules, depending upon the tissue from which they were obtained (23, 24). Thus, in the case of liver and pancreas the large granules were considered to be mixtures, because of the secretory function of these tissues, while in the case of a lymphosarcoma the large granule preparation was considered to consist of mitochondria. Hogeboom, Schneider, and Palade (60) found, however, that the large granule fraction isolated from homogenates of rat liver in 0.88 M sucrose consisted entirely of morphologically and cytologically unaltered mitochondria. This was shown by the fact that many of the isolated granules were elongated in form, a characteristic of intracellular mitochondria, and also by the fact that all the granules in the preparation were stained vitally by Janus Green B, the generally accepted stain for intracellular mitochondria. Furthermore, none of the isolated granules was stained with neutral red, a stain widely used for the demonstration of secretory granules, although the latter could readily be demonstrated in unbroken liver cells present in the homogenate.

The procedure for the isolation of mitochondria consists of the following steps: (*a*) disruption of cell membranes (Potter-Elvehjem homogenizer [107]) in a large volume of a suitable medium, (*b*) sedimentation of nuclei and unbroken cells at low speed, and (*c*) sedimentation of mitochondria at higher speed. The medium in which the homogenization is made has a profound effect on the morphological, cytological, and biochemical properties of the isolated mitochondria, as well as on their yield. If distilled water is used, the mitochondria swell to enormous size and eventually burst if maintained in water for a sufficient length of time (21, 24). The latter finding is not of great importance to the isolation of mitochondria, however, because the isolation can be completed long before appreciable lysis occurs. Of more importance is the fact that the biochemical properties of the mitochondria have been altered. Thus, liver mitochondria isolated from water homogenates contain high concentrations of cytochrome c, but the cytochrome c is unable to function in the oxidation of succinic acid by the mitochondria (128). Mitochondria isolated from water homogenates are also unable to oxidize octanoic acid (71). In isotonic solutions, both intracellular mitochondria and mitochondria released from ruptured cells are

[1] Experiments in progress at this writing have demonstrated conclusively that the distribution of DNA and of structures resembling chromosomes in liver nuclei was much different in sucrose solution than in isotonic saline solution. In sucrose, the nuclei were homogeneous in appearance under the microscope, while in saline the nuclei showed the typical pattern of precipitated chromatin (cf. 118). When the nuclei in sucrose were disrupted, chromosome-like structures were not visible, and over 60 per cent of the DNA of the nuclei was soluble; i.e., nonsedimentable at 60,000 *g*. In saline, however, structures similar to the chromosomes described by Mirsky and Ris (91) were released from the nuclei, and 92 per cent of the DNA was sedimented at the low speed (5,000 *g*) required to segregate these structures. These experiments would appear to indicate that DNA is not associated with structures comparable to chromosomes but is colloidally dispersed within the resting nucleus.

[2] Chantrenne (18) has reported experiments which indicated that the cytoplasm of the liver cell was a heterogeneous collection of particles of varying size, enzymatic activity, and chemical composition, and he concluded that the separation of liver particles into large granules (mitochondria) and microsome fractions was a partly arbitrary procedure. Since these liver homogenates were made in electrolyte solutions, however, it seems probable that a large proportion of the large granules were lost during the initial centrifugation used to prepare the nuclei and cell-free extracts employed in the experiments (Chantrenne, personal communication). The fact that microsomes are also aggregated by electrolytes (61, 66) would also have a bearing on these experiments, and it would appear that the question of heterogeneity of cytoplasmic particles requires reinvestigation.

morphologically altered, as shown by the fact that the mitochondria are predominantly spherical in shape (60). In addition, in isotonic *salt* solutions, the mitochondria do not stain vitally with low concentrations of Janus Green B and are agglutinated to an extent sufficient to prevent their adequate separation from the nuclei (60). Thus, losses of mitochondria as great as 80 per cent have been reported when isotonic saline solutions were used (112, 122). In addition, the contamination of the mitochondria by aggregated microsomes has also been reported (66). The agglutination caused by electrolytes is largely avoided by the use of either hypertonic or isotonic sucrose solutions (60). In addition, the mitochondria isolated in hypertonic sucrose retain their ability to stain vitally with Janus Green B and possess the morphological characteristic of intracellular mitochondria—namely, an elongated shape (60). The use of hypertonic sucrose has the disadvantage, however, of causing inhibition of the activity of several enzyme systems (72, 78, 125). Comparative studies of the distribution of enzymes in rat liver fractions obtained from both hypertonic and isotonic sucrose homogenates have failed to demonstrate any substantial differences between the two sucrose concentrations (129), and it has been concluded that for biochemical studies the use of isotonic sucrose is preferable at the present time (61, 127, 129). The use of the latter has the added technical advantage that the fractionations can be completed in much less time and at much lower centrifugal forces than are required by the greater viscosity and density of the hypertonic sucrose homogenates.

Several modifications have been proposed for the isolation of mitochondria from hypertonic sucrose homogenates. Kennedy and Lehninger (71) have suggested the addition of KCl to the nuclei and cell-free liver extract to agglutinate the mitochondria and thus to permit their isolation at a much lower centrifugal force. The permissibility of such a procedure is questionable, since, in our experience, electrolytes produce agglutination of submicroscopic particles as well as of mitochondria (61, 66). Evidence that this procedure does not yield a mixture of the two types of particles would require data on the total nitrogen and PNA content of this fraction. Thus, the ratio of PNA to total nitrogen would permit a decision as to whether this fraction was contaminated with submicroscopic particles, since this ratio is much higher for liver submicroscopic particles than for liver mitochondria.

Similar criticisms might be made of the modifications employed by Leuthardt and Müller (80). In their procedure, the homogenate is made in isotonic KCl and centrifuged at 1,500–2,000 *g*. The sediment is then resuspended in isotonic mannitol and recentrifuged. The mitochondria remain in the supernatant and are removed. It would appear that some submicroscopic material may also be present in the supernatant fluid.

Cunningham *et al.* (31) have fractionated frozen liver in a solution containing 0.88 M sucrose, 0.14 M NaCl, and 0.01 M phosphate buffer. They state that the use of unfrozen tissue or the omission of salt from the medium did not affect the amount of nitrogen or nucleic acid in the mitochondrial fraction. An examination of their data, however, revealed several facts that appear to disagree with this statement, e.g., the large amounts of nitrogen in the nuclear fraction and of PNA in the mitochondrial fraction, the low amounts of nitrogen and PNA in the microsome fraction, and the large amount of PNA in the supernatant or soluble fraction. The fact that Kennedy and Lehninger (71) were able to add salt to 0.88 M sucrose extracts of liver to cause aggregation of mitochondria would also appear to disagree with the findings of Cunningham *et al.*

As discussed later in this review, the yield of mitochondrial material with the sucrose method represents about 25 per cent of the total nitrogen of the whole liver tissue (60, 125, 130). The yield has not been determined in terms of number of mitochondria, although this would appear to be a project of considerable importance in view of the fact that the amount of mitochondrial material may increase or decrease in various physiological and pathological conditions (116, 140). The yield of mitochondria can also be expressed in another manner, namely, in terms of enzymatic activity that appears to be associated exclusively with the mitochondria. Thus, in the case of cytochrome oxidase, which appears to be localized exclusively in the mitochondria, the yield would appear to be as high as 80 per cent (122, 130). In regard to the purity of the fraction, both chemical studies and studies with the light, dark field, phase, and electron microscopes have indicated that the fraction is free from contamination (26, 32, 60).

One point in the procedure for the isolation of mitochondria that has not been described sufficiently is the removal of submicroscopic material that sediments along with the mitochondria. Muntwyler *et al.* (97) have recently called attention to difficulties in the preparation of the mitochondrial fraction occasioned by the presence of incompletely sedimentated material above the mitochondrial pellet. These authors reached the conclusion that this material belonged to subsequent fractions, since microscopic examination re-

vealed the presence of relatively few mitochondria. The reviewers have also observed the presence of this partially sedimented material and have always removed it from mitochondria, since it was found to be submicroscopic in nature on the basis of both microscopic examination and biochemical properties. On only one occasion (127), however, have the reviewers mentioned the importance of separating it from mitochondria. The appearance of this material is considerably different from that of the mitochondrial pellet, since it is pink-white in color, in contrast to the tan color of the mitochondria. This difference in color, coupled with the fact that it is not firmly packed, makes its removal from the mitochondria quite simple. The separation is most easily made after the second sedimentation of mitochondria, since the differences between the mitochondria and the submicroscopic particles are best seen at this stage. Failure to remove this submicroscopic material from the mitochondrial fraction results in rather obvious redistributions, such as the presence of too much nitrogen and PNA in the mitochondria and too little nitrogen and PNA in the submicroscopic particles, and may account for the high concentrations of PNA in the mitochondrial fraction reported by other workers (31, 111–116).

Secretory granules.—The isolation of secretory granules from liver and pancreas was reported by Claude (19–21). However, Lazarow (77) and Hoerr (54) argued that the secretory granules of Claude were actually mitochondria. Furthermore, Hogeboom *et al.* (60) have found that this fraction in rat liver was composed entirely of mitochondria and that a large proportion of the secretory granules apparently disintegrated when the cells were broken. In more recent publications, Claude (23–25) states that the secretory granules or large granule fraction consists mainly of mitochondria mixed with unknown proportions of secretory granules. According to the experiments of Palade and Claude (101, 102), granules that apparently correspond to secretory granules are present in homogenates of liver in water, saline, or hypertonic sucrose. When homogenates in the latter medium are fractionated, these granules migrate centripetally and collect in a lipid layer at the top of the centrifuge tube. In addition to the secretory granules, this lipid layer also contains neutral fat droplets in large numbers. It is not clear whether the secretory granules present in the lipid layer constitute the entire complement of secretory granules present in the whole tissue. In the case of the homogenates of liver in water and in saline, the secretory granules did not appear in this lipid ayer, and the fate of the granules in these media remains to be clarified. From this discussion it would appear that adequate methods for the isolation of secretory granules remain to be developed, and, in future studies, the question of the stability of the secretory granules would appear to be a primary concern. However, the use of hypertonic sucrose would appear to provide a point of departure for further experiments. Isolation of the lipid layer from sucrose homogenates, followed by its transfer to other media of lower density, might be suggested as a means of testing both the stability of the granules and also of separating them from the fat droplets with which they are contaminated.

Isolation of melanin granules.—Melanin granules have been isolated from amphiuma liver (25), from the ciliary processes of beef eyes (52), from mouse and rat melanomas (25, 142), and from frog eggs (117) by procedures similar to those employed for the isolation of mitochondria. The size and density of the melanin granules are, in fact, similar enough to those of mitochondria to raise the question of mitochondrial contamination. Such contamination has apparently been ruled out in the case of the melanin granules isolated from amphiuma liver, since photomicrographs and electron micrographs of these particles fail to show the presence of other particulate material (25). That the mitochondria of frog eggs can be separated from melanin granules was clearly shown by Recknagel (117).

In the experiments with the other tissues, however, this had not been demonstrated as convincingly, and in the case of the melanomas conflicting reports have appeared on the question of the simultaneous presence of mitochondria and melanin granules in the cells of this tissue. Thus, evidence has been presented to show that the melanin granules possess the staining characteristics usually considered specific for mitochondria and that these melanin granules were the only granules with the cytological properties of mitochondria in the melanoma cells (142). On the other hand, particulate structures present in melanoma cells have been identified provisionally as mitochondria (32). These bodies are morphologically different from the melanin granules, are unpigmented, and are so small that some of them cannot be resolved in the light microscope.

Isolation of particulate glycogen.—Glycogen is present in liver homogenates in submicroscopic form and sediments at centrifugal forces intermediate between those required for mitochondria and submicroscopic particles. Particulate glycogen has been isolated by Lazarow (76, 77) and by Claude (24).

Isolation of submicroscopic particles (*micro-*

somes).[2]—Microsomes were first recognized and isolated by Claude (19). These particles were at first considered to be mitochondria but were subsequently renamed microsomes, in view of the facts that they were submicroscopic in size and that the larger, microscopically visible particles had been identified as mitochondria. The isolation of microsomes is accomplished by the high speed centrifugation of extracts that have been cleared of nuclei, cells, mitochondria, and glycogen in previous centrifugations.

There has been considerable discussion whether the microsomes exist as such within the intact cell or whether they are produced during or after cell rupture. This question is difficult to answer, since these particles are too small to be seen in living cells. Claude (21), however, has presented evidence to show that the microsomes possess the same staining characteristics as the so-called ground substance of the cytoplasm. On the other hand, Lagerstedt (74), in an extensive study of livers of normal and starved animals, reached the conclusion that the microsomes obtained by cell fractionation are probably breakdown products of the basophilic inclusions present in the cytoplasm of fixed liver cells. It seems possible, however, that these inclusions may themselves be aggregates produced by the cytological procedures. Although the exact derivation of microsomes is thus not clearly established, studies with the electron microscope (27, 106) have revealed the presence of cytoplasmic structures too small to be visible in the optical microscope. The size of these structures is of the same order as that of microsomes, as estimated for the latter by their behavior in the centrifuge.

Procedure for the complete fractionation of tissues.—In the past, the tendency has been to concentrate on the isolation of a single tissue fraction and to discard the remainder of the tissue and ignore it entirely or to estimate the properties of the remainder by difference. Although such a policy may be of value in gaining an idea of the properties of a given cell fraction, from the standpoint of the cell as a whole the procedure may lead to an entirely erroneous impression of the role played by a given portion of the cell. To obtain information on the contributions made by each portion of the cell, comprehensive fractionation procedures have been devised and applied to various normal and tumor tissues (55, 58, 60, 61, 103, 122–125, 129–132). These methods permit the separation of the tissue into four fractions: a nuclear fraction, a mitochondrial fraction, a submicroscopic particulate or microsome fraction, and a supernatant or soluble fraction. The preparation and properties of the first three fractions have been already described in previous paragraphs. The soluble fraction is merely the supernatant fluid remaining after removal of nuclei, mitochondria, and microsomes and contains, in addition to soluble material, lipid droplets that have migrated centripetally.

The Results of Studies of Isolated Tissue Fractions

Nuclei.—No adequate determinations have been made of the proportions of the tissue mass that is represented by the nuclei. This is largely due to the fact that most studies on nuclei have dealt with their isolation in a "purified" state without attention to yield. On the other hand, studies in which the nuclei have been isolated quantitatively from the tissue have failed to yield a cytologically pure fraction. However, investigations of the latter fraction do permit an estimate of the nuclear content of tissues. Thus, present results indicate that less than 15 per cent of the total nitrogen of rat (129) and mouse (130) liver homogenates and 23 per cent of rabbit liver (79), rat kidney (132), or mouse hepatoma (130) nitrogen is present in this fraction. Values as low as 5 per cent of the total protein of mouse liver have been reported for nuclei isolated with citric acid (8) but from the low protein-DNA ratio reported for these nuclei, it seems clear that they had lost a considerable amount of their original protein (105). Marshak (83) has reported that the nucleus of the mouse liver cell occupies only 6 per cent of the cell volume, on the basis of cytological measurements. However, the ratio of cytoplasmic volume to nuclear volume on sections of rat liver indicated that the nuclear volume was about 15 per cent of the total cell volume (121). More recently, Mirsky and Ris (93) stated that nuclear volumes in their liver preparations ranged from 10 to 18 per cent. It would appear that the total nitrogen content of the nuclear fraction approximates that expected from the latter two groups of cytological determinations.

The distribution of DNA has been studied in rat (60, 112, 122, 125, 129), rabbit (79), and mouse liver (131); rat kidney (122, 132); normal and leukemic mouse spleen (103); primary rat liver tumors (115, 123); and mouse hepatomas (131). The results of these studies have demonstrated that essentially all the DNA present in the tissues is recovered in the nuclear fraction. These findings provide confirmation of cytological studies which have indicated that DNA is exclusively a nuclear constituent. Cases (115, 131) in which the entire tissue DNA was not recovered in the nuclear fraction can probably be explained on other grounds,

e.g., fragmentation of nuclei during the isolation procedure or the presence in the original whole tissue of nuclear fragments resulting from the disintegration of nuclei.

Although the nuclear fraction obtained by the systematic fractionation of tissues is admittedly a mixture of nuclei with some mitochondria and unbroken cells, the results that have been obtained with this fraction in certain comparative studies have been sufficiently striking to eliminate nonnuclear contaminants as a source of great error. Thus, it was observed several years ago that the nuclear fraction of primary rat liver tumors induced with 4-dimethylaminoazobenzene (DAB) contained much larger amounts of DNA and of dry material than did the nuclear fraction of normal liver (123). The data therefore implied that the liver tumor contained more nuclei per volume of tissue than did normal liver, since the increase in DNA in the liver tumor was paralleled by an increase in dry weight. This view was supported by determinations of the ratio of cytoplasmic volume to nuclear volume in sections of the two tissues (121). The latter studies indicated that this ratio was much greater for normal liver than for liver tumor, and the magnitude of the difference was approximately sufficient to account for the increased amounts of DNA and dry weight in the nuclear fraction of the tumor. These findings were confirmed and extended by Price *et al.* (113–116), who were able to show that the DNA and protein content of the nuclear fraction obtained from the livers of rats fed carcinogenic azo dyes increased after only short periods of feeding and long before tumors appeared. They were also able to show that the number of nuclei increased in proportion to the increase in DNA, with the result that the amount of DNA per nucleus remained approximately constant (116). Thus, it appeared from these studies that the carcinogenic process in liver and increased cellularity were intimately related. More recent work, however, indicates that this increased cellularity is apparently much greater in azo dye than in other types of carcinogenesis, because in the case of liver tumors induced with acetylaminofluorene (48) and of a transplantable mouse hepatoma (131) the DNA content (and presumably the degree of cellularity) was only slightly increased above that of control livers. Furthermore, in the case of normal and leukemic spleens (103), the DNA content of the leukemic spleen was somewhat lower than that of the normal spleen. A possible explanation for the findings in azo dye carcinogenesis is extensive proliferation of bile duct epithelium.

The presence of PNA in the liver nucleus appears to be definitely established, but its exact concentration remains to be determined. The strongest evidence for the presence of PNA in the nucleus comes from studies with radioactive phosphorus, which have demonstrated that the PNA present in isolated nuclei has a much higher turnover rate than the PNA associated with other fractions of the liver cell (68, 84). Recent analyses by Dounce *et al.* (37) on nuclei isolated from rat liver by means of the Behrens' procedure (9) indicate that the PNA concentration in nuclei may be much greater than was previously supposed. PNA concentrations 1.27 and 1.78 times as great as the DNA concentrations were obtained in two separate experiments. On the assumption that all the DNA was present in the nuclei and that the DNA and PNA contents of the liver were 250 and 1,000 mg. per cent, respectively (121), this finding would mean that 32–45 per cent of the total PNA was present in the nucleus. This would appear to be much too high in view of other studies in which it was found that about 80–90 per cent of the total liver PNA was present in the mitochondrial, submicroscopic particulate and supernatant fractions (60, 125, 131, 132). The possibility that the nuclei isolated by the Behrens' procedure could be contaminated by submicroscopic material was considered to be improbable by these authors (37) in view of the method used for the isolation of the nuclei. However, another possibility that should be considered is that the submicroscopic particles or the PNA they contain are actually localized in the liver nucleus and are released during the isolation procedures in aqueous media. If such is the case, it is certainly remarkable that the presence of such large amounts of PNA in the nucleus has escaped the attention of the cytologist.

The lipid content of rat liver nuclei has been studied by Dounce (35), who found that the amount of lipid present depended upon the pH at which the nuclei were prepared. Thus, at pH 6 the nuclei contained as much as 10.8 per cent lipid, while at pH 4 they contained only 3.2 per cent. The low values were considered to be more nearly correct, because, according to Dounce (35), nuclei prepared at this pH do not lose either DNA, lipid, or protein. The data do not entirely support this statement, however, because in several instances the DNA contents of nuclei prepared at the two pH's did not differ markedly. Further work will be required to clarify this point. In regard to the nature of the lipid associated with nuclei, it would appear that phospholipid was absent, because Dounce *et al.* (37) have reported that all the phosphorus of isolated nuclei was accounted for by acid-soluble and nucleic acid phosphorus.

The occurrence of free amino acids in nuclei isolated by the Behrens' technic was studied by the use of paper chromatographic procedures (37). The distribution of amino acids in isolated nuclei was similar to that obtained with whole liver cells.

The association of enzymes with isolated nuclei has been studied by Dounce and his associates and reviewed previously (36). Most of these studies have been made with nuclei isolated in dilute citric acid, although some have employed the nuclei isolated with the Behrens' technic. Interestingly enough, the enzymatic activity of the latter nuclei was as great as that of the former, in the case of two enzymes studied (37). The enzymes that have been found to be associated with nuclei include aldolase, D-amino acid oxidase, arginase, catalase, cytochrome oxidase, enolase, esterase, acid and alkaline phosphatases, phosphorylase, lactic dehydrogenase, and uricase. With the exception of alkaline phosphatase, the concentration of the enzymes in the isolated nuclei was the same as or lower than the concentration in the whole tissue. The concentration of alkaline phosphatase in the isolated nuclei was about twice as great as in the whole tissue. It is not clear what significance can be attached to the association of these enzymes with nuclei, inasmuch as the total enzymatic activity present in the nuclei usually represented only a small fraction of the total tissue activity. Thus, in the case of cytochrome oxidase, studies with the nuclear fraction have shown that as little as 5 per cent of the total liver activity is present in this fraction (122). In view of the fact that 75–80 per cent of the total liver activity has been found to be associated with the mitochondria and that the latter are contaminants of the nuclear fraction (122, 130), it would appear that the total activity of the nucleus may well be much less. Thus, as has been pointed out in earlier paragraphs, the possibility that enzymes were adsorbed on the nuclei or that the nuclei were contaminated must be considered in view of the small amount of enzymatic activity present in the nuclei and the fact that the enzyme concentrations in the nuclei were less than, or the same as, those in the whole tissue.

Studies on other enzymatic properties of the nuclear fraction have indicated that nuclei may contain high concentrations of certain phosphatases. Thus, high concentrations of ATP-ase and AMP-ase have been reported for the nuclear fraction isolated from rat liver (98, 122), rat liver tumor (123), mouse liver, and mouse hepatomas (131). In the case of AMP-ase, the nuclear fraction accounted for as much as 45 per cent of the total activity of rat liver (98), while, in the case of ATP-ase, somewhat lower percentages were present in the nuclear fraction.

Chromosomes.—Chromosomes have been isolated from a number of tissues, including thymus (91–93), a rat lymphosarcoma (28), normal and leukemic mouse spleen (104), normal and hyperplastic epidermis, and a squamous-cell carcinoma (45). One of the major constituents of the chromosomes is DNA. In the case of the first four tissues mentioned, the DNA content of the chromosomes was 37 per cent (28, 92, 104). On the other hand, the DNA content of the chromosomes obtained from normal and hyperplastic epidermis was only about 12 per cent, while that of the squamous-cell carcinoma was approximately 21 per cent (45). No data have been reported which permit an estimate of the proportion of the total tissue DNA that is associated with the chromosomes. Thus, although it is apparently established that the entire DNA of the tissue is present in the nuclei, it is not clear whether DNA can exist in the nucleus apart from the chromosomes.[1] The latter problem would appear to be of considerable importance from the standpoint of the postulated genic properties of DNA.

PNA has also been found to be present in isolated chromosomes but in considerably lower concentrations than DNA (104). The association of PNA with isolated chromosomes must be considered in light of the fact that the chromosomes contain adhering nucleoli which are known to contain high concentrations of PNA. The amount of PNA accounted for by the nucleoli remains to be established, and the low concentrations of PNA in the chromosomes also need to be assessed in regard to the high concentration of PNA found by Dounce *et al.* (37) in nuclei isolated by the Behrens' procedure.

Proteins represent another major component of isolated chromosomes and are of two types in the case of mammalian chromosomes (85, 92). The main protein is of the histone type, although a smaller amount of an acidic protein is also present. Little is known concerning possible enzymatic functions of chromosomal proteins.

Chromosomes have been fractionated by means of 1 M NaCl into a soluble portion and a residue possessing the morphological properties of the original chromosomes (91–93). The soluble fraction contains most of the DNA and histone, while the residual chromosome contains PNA, some DNA, and the nonhistone protein. In the case of the residual chromosomes obtained from leukemic spleen, the PNA content was about twice as great as that of the residual chromosomes of normal spleen (104). It has also been stated that the entire

alkaline phosphatase activity of thymus chromosomes is localized in the residual chromosome (89).

Mitochondria.—The use of data obtained by the cell fractionation technic in estimating the proportion of the cell mass represented by mitochondria is somewhat complicated by the fact that none of the procedures used in the homogenization of tissues allows disruption of all cells. A variable proportion of the mitochondria is thus present in the intact cells of the nuclear fraction. Another factor influencing the yield of mitochondria is that complete separation of free mitochondria from nuclei is not readily obtained. The data obtained from cell fractionations involving cell disruption with the Potter-Elvehjem homogenizer and carried out in solutions of nonelectrolytes (to avoid aggregation of particles) probably provide a basis for the most reliable estimates of the mitochondrial content of tissues.

In experiments carried out with sucrose solutions (either 0.25 M or 0.88 M) as media, the mitochondrial fraction isolated from rat liver accounted for 23–26 per cent of the total nitrogen (60, 125, 130, 132) and 30–33 per cent of the total protein (112, 115) of the original homogenate. The mitochondria of C3H mouse liver contained 24 per cent of the original total nitrogen (130). A much lower value (11 per cent of the total nitrogen) was obtained with rabbit liver (79). Somewhat lower values have also been obtained with such media as water (122, 123, 128) and isotonic NaCl (6, 66, 99, 128).

In view of evidence that mitochondria probably contain all the succinoxidase activity of the liver cell (60), it is possible to determine roughly the amount of mitochondrial material in the nuclear fraction from the succinoxidase activity of the latter. On this basis it can be estimated that mitochondria account for 30–35 per cent of the total nitrogen of rat or mouse liver.

A finding of interest is that rat and mouse hepatomas contain considerably less mitochondrial material than does normal liver (115, 123, 130). In this respect, Price *et al.* have noted a decline in the mitochondrial protein of livers of rats fed 4-dimethylaminoazobenzene (DAB) and a number of derivatives of DAB for periods insufficient to produce tumors (114, 116). Furthermore, the extent of this decline was roughly proportional to the carcinogenicity of the compound fed. A toxic but noncarcinogenic derivative, 2-methyl-4-dimethylaminoazobenzene (2-MeDAB) had the remarkable effect of producing a considerable rise in the protein of the mitochondrial fraction (109, 116).

Relatively few data are available showing the mitochondrial content of other tissues. Kidney mitochondria accounted for 17 per cent of the dry weight and 20 per cent of the total nitrogen of the whole tissue (122, 132). The mitochondria of the Flexner-Jobling rat carcinoma contained 8 per cent of the total nitrogen of the tumor (79). Values of 2.5 and 2.2 per cent were found by Petermann *et al.* (3, 103) for the mitochondrial nitrogen of normal and leukemic mouse spleen. These extremely low values are probably a reflection of the small number of mitochondria in the cells of lymphoid tissue and of the fact that a considerable proportion of the cells were not disrupted by homogenization (3, 103).

The PNA content of isolated mitochondria has been determined by a number of investigators. Most of the results obtained with rat and mouse liver indicate that the concentration of PNA in mitochondria is somewhat lower than in whole liver and much lower than in submicroscopic particles. It has been shown that the PNA content of mitochondria declines on repeated sedimentation, as a result of the removal of submicroscopic particles, and eventually reaches a constant level after three or four sedimentations (60). Thus, the PNA content of mitochondria can be used as an index of the degree of contamination of this fraction with submicroscopic particles.

A number of determinations (55, 60, 97, 122, 125, 132) have yielded an average value of 11 (range: 7 to 13) μg. of PNA phosphorus per milligram of total nitrogen for rat liver mitochondria isolated in sucrose solutions, as compared with a value of 27 (range: 23 to 30) for the original whole tissue and 63 (range: 46 to 77) for submicroscopic particles. The proportion of the total PNA of whole rat liver present in mitochondria appears to be approximately 15 per cent.

Results obtained with the mitochondria of mouse liver (131), rabbit liver (79), and rat kidney (122, 132) for the most part indicate a concentration of PNA in the same general range as that obtained with rat liver. Barnum and Huseby (6) found a somewhat high value for mouse liver mitochondria; later, however, these investigators pointed out that considerable amounts of submicroscopic particulate material contaminated their mitochondrial preparation, presumably because of the use of 0.85 per cent NaCl as the medium for fractionation (66). Ada (2) reported a very high value for rabbit liver mitochondria and did not confirm the unequal PNA distribution between mitochondria and submicroscopic particles previously noted by others. Ada's results are open to question, however, because they were not obtained by direct PNA determinations but on the assumption that only PNA phosphorus remained

after extraction of the preparations with ethanol-ether and ether.

The concentration of PNA in the mitochondria of tumors derived from the livers of rats and mice is, in general, higher than the concentration of PNA in normal liver mitochondria (115, 123, 131). The proportion of the PNA of whole tissue recovered in the mitochondrial fraction is, however, less in hepatomas than in normal liver because of the great decrease in the amount of mitochondrial material in the tumors. In this respect, Price *et al.* (114, 116) found that the decline in the total amount of PNA recovered in the mitochondrial fraction obtained from rats fed DAB and related compounds generally followed the decline in the total protein of the fraction. Of additional interest in these experiments was the finding that 2-MeDAB, which caused an increase in mitochondrial protein, produced a considerable decrease in mitochondrial PNA. The PNA content of mitochondria isolated from leukemic spleen was not greatly different from that of normal spleen mitochondria (3, 103).

It should be pointed out that the relatively low PNA concentration in mitochondria brings up the question whether the PNA found in the fraction may be due entirely to submicroscopic particles not completely removed by repeated sedimentation of mitochondria. This is a difficult question to answer in view of the established principle that the presence of a substance in a cell fraction in a lower concentration than in the original whole tissue should be interpreted with caution. Certain other observations, however, including electron microscopical evidence for the homogeneity of the mitochondrial fraction (60), the effect of ribonuclease on mitochondria (144), and their staining properties (60), indicate that mitochondria contain PNA.

A few studies have been made of the lipides of mitochondria (2, 6, 24, 122, 123). In general, the results indicate that 25–30 per cent of the dry weight of liver mitochondria consists of lipides, of which approximately two-thirds is present in the form of phospholipide. It may be mentioned that the concentration of lipide, like that of PNA, is considerably lower in mitochondria than in submicroscopic particles.

The association of enzyme activity with mitochondria has been the subject of extensive investigations in the last few years. As indicated in a previous review (126), many of the earlier data were obtained from experiments involving the isolation of a mixture of mitochondria and submicroscopic particles (13, 17, 46, 135). In addition, both these and many later results are open to the serious objection that not all fractions of the tissue were analyzed, and it is therefore not possible to draw up balance sheets or to compare the enzymatic activity of mitochondria with that of the original whole tissue. In short, so many enzymatic functions have been ascribed to the mitochondrion that the remainder of the cell would almost seem to be excess baggage.

The most striking and the earliest discovered enzymatic property of mitochondria is their content of cytochrome oxidase and certain related respiratory enzyme systems. It has been clearly demonstrated, for example, that by far the majority of the cytochrome oxidase and succinoxidase of rat (56, 60, 122, 123, 128) and mouse (130) liver and of rat kidney (122) is recovered in the mitochondrial fraction (cf. 15), and it seems entirely likely that the small amount of activity shown by other fractions is the result of contamination with mitochondria (60). Other respiratory enzyme systems concentrated in liver mitochondria are octanoic acid oxidase (70, 71, 125) and oxalacetic acid oxidase (132). In both of these systems, however, the activity of mitochondria is enhanced by the addition of other fractions which alone have little or no activity. The enhancement is slight in the case of octanoic acid oxidase and pronounced in the case of oxalacetic oxidase, both submicroscopic particles and final supernatant having the effect. The fact that mitochondria are capable of oxidizing oxalacetate is of particular interest, since it is an indication, in addition to their content of cytochrome oxidase and succinoxidase, of their participation in the Krebs cycle reactions. The role played by other cell fractions in the oxidation of oxalacetate, together with recent data on the oxidation of *d*-isocitrate (61), makes it clear, however, that mitochondria are not *solely* responsible for the Krebs cycle reactions.

Additional enzyme systems associated with liver mitochondria are DPN-cytochrome c reductase (55, 58) and TPN-cytochrome c reductase (61). It may be noted that both these systems are also present in submicroscopic particles, DPN-cytochrome c reductase being concentrated to a greater extent in the latter fraction than in mitochondria. The other system is more concentrated in mitochondria. Several phosphatases are also concentrated in the mitochondrial fraction of liver; these include acid phosphatase (98), AMP-ase (98), and ATP-ase (98, 122, 131). A large proportion of the uricase activity of whole liver was also recovered in the mitochondria (120).

The system capable of synthesizing *p*-aminohippuric acid (PAH) was recovered almost in its entirety in the mitochondrial fraction (72). In

these and other experiments (110) it was also found that mitochondria effectively maintained high levels of ATP under conditions optimal for the synthesis of PAH. More recently, it has been shown that the particles, in the presence of an oxidizable substrate such as glutamate or α-ketoglutarate, are capable of synthesizing ATP from AMP at a rapid rate.[3] The presence of respiratory enzymes in mitochondria and their ability to carry out aerobic phosphorylation are, of course, indicative of an enzyme organization capable of supplying energy for other synthetic reactions, including the synthesis of peptides and proteins. Although recent work has indicated that mitochondria can incorporate glycine and lysine into proteins (12), the significance of such findings with respect to the participation of mitochondria in the synthesis of proteins is not as yet clear.

Cytochrome c (128, 129), vitamin B_6 (113), and riboflavin (112) are concentrated in mitochondria to a considerable extent, the latter finding indicating the probable presence of flavoproteins not as yet studied. Catalase was found in mitochondria but not in appreciably greater concentration than in whole liver (42). Most of the catalase activity was recovered in a supernatant containing submicroscopic particles and soluble material.

A number of other enzymes and related compounds have been detected in suspensions of liver mitochondria. Further work will be necessary to clarify the situation with respect to these findings, however, because complete recovery data are not available. Among the enzymes found are D-amino acid oxidase (22), α-glycerophosphate dehydrogenase (22), ribonuclease (22), citrullin synthesis (96), "myokinase,"[3] and the oxidation of citrate (71), α-ketoglutarate (71), and glutamate (72). Recently, Hird and Rowsell (53) have reported that the insoluble particle fraction of rat liver homogenates contained all the glutamate-phenylpyruvate transaminase activity of the whole tissue and was also able to catalyze the formation of tyrosine, alanine, and aspartate from the corresponding keto-acids. The supernatant catalyzed transamination between glutamate, aspartate, and alanine only. According to these investigators (53), mitochondria isolated in 0.88 M sucrose were found to be active in promoting the reactions found in the insoluble particle fraction. Other workers have also detected vitamin A in preparations of mitochondria (41, 43).

The enzyme content of the mitochondria of liver tumors is markedly different in several respects from that of normal liver mitochondria. Thus, the specific succinoxidase, cytochrome oxidase, and ATP-ase activities of rat liver tumor mitochondria were 3–5 times lower than the corresponding specific activities of normal rat liver mitochondria (123). A similar picture for these three enzyme systems has been obtained in studies of C3H mouse liver and C3H mouse hepatoma 98/15 (130, 131). DPN-cytochrome c reductase, on the other hand, was found in the latter studies to be present in much higher concentration in hepatoma mitochondria than in liver mitochondria (58). The synthesis of PAH, a function of normal liver mitochondria, is carried out by hepatoma 98/15 homogenates and mitochondria at a negligible rate (72). Whether the latter finding is a reflection of the absence of the PAH-synthesizing enzyme itself or the result of the inability of the tumor mitochondria to maintain ATP levels (a condition necessary for PAH synthesis) is an interesting question. The extremely low activity of tumors in the oxidation of oxalacetate (108) and octanoate (5) is probably also a reflection, at least in part, of a defect in the mitochondria. Recent studies of the amino acid composition of the mitochondria of a number of tissues, including several tumors, revealed no qualitative differences (81). The amino acid composition of the *total proteins* of the nuclear, mitochondrial, microsomal, and supernatant fractions has been studied by Schweigert *et al.* (133). These workers observed several differences in the amino acid composition of the proteins obtained from the fractions of normal liver, the livers of animals fed the carcinogen, DAB, and the liver tumors induced by DAB.

Green *et al.* (47) have described an enzyme suspension made by preparing homogenates (by means of the Waring Blendor) of various tissues (e.g., liver, kidney, heart) in isotonic KCl, centrifuging the preparation at 2,000 *g*, and washing the sediment several times in the same medium. This sediment (cf. the washed liver residue of Lehninger and Kennedy [78] and Cohen and McGilvery [29]) has been shown to catalyze the complete oxidation of pyruvic acid, fatty acids, and amino acids to CO_2 and H_2O through the citric acid cycle, and has been given the name "cyclophorase" to indicate that it is an integrated enzyme complex and thus, presumably, a cellular entity. The mode of preparation of cyclophorase is such as to indicate the presence of whole cells, nuclei, nuclear fragments, mitochondria, mitochondrial fragments, and submicroscopic particles, the latter probably being present as a result of aggregation in the presence of KCl (61, 66). Harman (49) has recently reported experiments designed to prove that cyclophorase is associated

[3] R. K. Kielley and W. W. Kielley, unpublished experiments.

with the mitochondria present in the sediment, and the terms cyclophorase and mitochondria are now used interchangeably (50, 64, 136). Since this conclusion by Harman has important cytochemical implications, it is felt that the evidence supporting it should be discussed in some detail.

A correlation of cyclophorase activity with mitochondria (49) was based on the following findings: (*a*) When nuclear fragments were separated from cell-free cyclophorase preparations, a portion of the activity (21–61 per cent), as indicated by oxygen uptake in the presence of α-ketoglutarate, remained. (*b*) It was stated that nuclear and "microsomal" elements of cyclophorase were incapable of oxidizing α-ketoglutarate. (*c*) Prolonged subjection of cyclophorase to the action of the Waring Blendor and of certain "transforming agents" resulted in disintegration of or damage to mitochondria and at the same time inactivated α-ketoglutarate oxidation. (*d*) An association of oxidative phosphorylation with the mitochondria of cyclophorase was demonstrated.

In the opinion of the reviewers, these findings do not constitute decisive proof that the cyclophorase activity of cyclophorase is associated with the mitochondria of the preparation. As mentioned previously, both α-ketoglutarate oxidation and oxidative phosphorylation have been demonstrated to occur in suspensions of mitochondria that have been freed from other cell constituents by differential centrifugation of liver homogenates (71, 72). These reactions can therefore be considered tests for mitochondria; they are not necessarily tests for cyclophorase, as *originally* defined, since complete oxidation of pyruvate to CO_2 and H_2O was not shown to occur. Thus, their use as tests for cyclophorase only leads to confusion concerning the definition of the term cyclophorase.

Another and perhaps more decisive indication of the association of cyclophorase activity with mitochondria would be the demonstration that the majority of the cyclophorase activity of *whole tissue* is recovered and concentrated in the mitochondrial fraction and that other cell fractions do not contribute to the activity of the whole tissue. That such is probably not the case has already been shown, however, by experiments involving the oxidation of the citric acid cycle substrates, oxalacetate (132) and *d*-isocitrate (61). An important point, which was demonstrated in the latter experiments with *d*-isocitrate (61) and which may have some bearing on the interpretation of the finding of a large number of oxidases (64) in cyclophorase, is based on the fact that cytochrome oxidase is localized, probably exclusively, in mitochondria (56, 122, 130). Thus, in the study of the intracellular distribution of any system ultimately dependent on cytochrome oxidase for oxygen uptake, the only fractions that could be expected to take up oxygen would be the mitochondria and the nuclei, the latter because of contamination by mitochondria. If the mitochondrial fraction contained only a small proportion of the total activity of a given dehydrogenase of the whole tissue (e.g., the isocitric dehydrogenase of liver [61]), the addition of the appropriate substrate would result in oxygen uptake, provided a large enough quantity of mitochondria was used. Results of this type can be very misleading from the cytochemical standpoint unless all fractions of the tissue are analyzed, a complete balance sheet drawn up, and unless the method of enzyme assay is a direct measure of the activity of the enzyme in question.

The finding that some enzymes (e.g., "lactic oxidase") are very readily removed from cyclophorase or occur largely in the supernatant after the first sedimentation of cyclophorase has been explained by Still and Kaplan (136) as the result of damage to mitochondria. It is further suggested that methods have not been devised for isolating mitochondria in their original state. In the reviewers' opinion, these findings can also be interpreted in another way, namely, that the enzymes in question are not associated with mitochondria, either within or outside the cell.

The gel-like consistency of cyclophorase has been considered a striking property of the preparation (64, 65, 136). In this respect, the reviewers have never observed gel-formation by mitochondria that have been washed numerous times with a variety of media. On the other hand, repeated homogenization and sedimentation of the nuclear fraction cause this pellet to swell and to assume a gel-like consistency. Thus, it seems possible that the gel-like properties of cyclophorase are due to the presence of nuclei and nuclear fragments rather than to mitochondria.

Recently, Harman (50) has questioned the evidence offered by a number of investigators for the existence of a mitochondrial membrane. Utilizing cyclophorase as a source of material, Harman has concluded that the behavior of mitochondria is compatible with a gel-like structure and does not require the presence of a semipermeable membrane. This conclusion is mainly based on an apparent lack of lysis of mitochondria in deionized water and a nonselective penetration of sodium and potassium ions. It may be pointed out that Harman's observations with respect to the lysis of mitochondria are directly contradictory to the observations of a number of other investigators (23, 24, 32, 56, 101). In addition, it appears doubt-

ful that a study of selective penetration of sodium and potassium ions is a crucial test for the presence of a membrane other than that of the erythrocyte. Finally, no explanation is offered for the fact that a structure having the morphological characteristics of a membrane has been seen repeatedly (26, 32, 66, 95) in electron micrographs of isolated mitochondria. Available evidence would seem, therefore, to be in favor of the existence of a mitochondrial membrane. It should be pointed out, however, that the mitochondrial membrane, if existent, is certainly unusual in its elasticity, for the particles will increase greatly in volume before lysing in hypotonic media (23, 24).

The probable existence of a mitochondrial membrane has brought up the question whether this membrane remains sufficiently intact after isolation of the particles to retain soluble compounds. Evidence for the integrity of the membrane was first indicated by the experiments of Claude (24), who found that after prolonged contact with distilled water the large granules of guinea pig liver underwent lysis, yielding particulate material of small size and a soluble fraction that included proteins and dialyzable compounds. More recently, it has been found that the disruption by means of sonic vibrations of the membranes of isolated rat liver mitochondria results in the release into solution of approximately 60 per cent of the total nitrogen (57). Furthermore, most of the nitrogen represents proteins that can be characterized on the basis of sedimentation constants in the analytical centrifuge. Similar results (59) have been obtained by disintegration of mouse liver mitochondria in an apparatus similar to that described by Milner *et al.* (88). Studies with the analytical centrifuge (59) also showed that three components of the mixture of proteins obtained from the mitochondria of C3H mouse hepatoma 98/15 corresponded in sedimentation constants to the proteins of normal liver mitochondria. A fourth component, present in the latter preparation, could not be detected in the hepatoma preparation.

In these experiments it was found that disintegration of mitochondria was accompanied by partial to complete inactivation of several complex enzyme systems (e.g., succinoxidase, octanoic acid oxidase) (57). Less complex enzymes were affected only slightly (57). These findings suggest that the functioning of a complicated enzyme system is dependent on a definite, spatial arrangement of its individual components and thus on the structural integrity of the mitochondrion.

Melanin granules.—The biochemical properties of melanin granules isolated from mammalian tissues have been the object of several studies. Hermann and Boss (52), utilizing the ciliary body as a source of material, found dopa (3,4-dihydroxyphenylalanine) oxidase, cytochrome oxidase, and succinoxidase activity in preparations of isolated pigment granules. No tyrosinase activity was detected. DuBuy *et al.* (38) made similar studies of the granules isolated from both melanotic and "amelanotic" melanomas of the mouse. Pigment-producing enzymes, both dopa oxidase and tyrosinase in one case and only dopa oxidase in another, were associated with the granules isolated from two pigmented melanomas. The colorless granules obtained from an amelanotic tumor did not possess dopa oxidase or tyrosinase activity. All preparations, however, contained cytochrome oxidase and succinoxidase.

The results of these two studies bring up the interesting question of a possible relationship between mitochondria and melanin granules (cf. the relationship between mitochondria and plastids [39]), since preparations of the latter showed respiratory enzyme activity characteristic of mitochondria. The possibility that the respiratory enzymes were present in the suspensions of melanin granules as a result of contamination by mitochondria was considered by duBuy *et al.* (38) and apparently ruled out by microscopic studies of the preparations obtained from the highly pigmented Harding-Passey melanoma. It was concluded that the data were consistent with the view that the melanoma granules represent a modified form of mitochondria. The possible presence of mitochondria too small to be seen in the optical microscope, however, arises from the results of studies by Dalton *et al.* (32).

The recent studies of Recknagel (117) may have some bearing on the problem. Utilizing mature eggs of the frog as a source of material, Recknagel was able to separate mitochondria from melanin granules and demonstrated that the cytochrome oxidase of the whole cells was associated with the former type of particle. Since these results were obtained from amphibian rather than mammalian tissue, however, they are not strictly comparable to the results reported by duBuy *et al.* (38).

Glycogen.—Particulate glycogen has been isolated from guinea pig livers by Lazarow (76, 77) and Claude (24). The actual yield of glycogen was 6.5 per cent of the total dry weight of the liver, but it was estimated that the probable concentration of particulate glycogen in the liver was 10–15 per cent (24). Particulate glycogen contains 92–93.5 per cent glycogen and 1 per cent protein according to Lazarow (76, 77). The nitrogen values reported by Claude (24) for particulate glycogen are in agreement with the latter. Claude also reported

that particulate glycogen contained sulfur in the same concentration as nitrogen. The significance of the presence of protein and sulfur in particulate glycogen is not known, but Lazarow (77) suggests that the protein may serve as a framework for maintaining glycogen in particulate form, because agents which disperse the glycogen markedly alter proteins.

Submicroscopic particles.—It is of interest that the submicroscopic particle (or microsome) content of several tissues approaches the mitochondrial content. In studies of rat liver fractions (60, 125, 132), 18–20 per cent of the total nitrogen was accounted for by submicroscopic particulate material. The microsomes of mouse liver accounted for 23 per cent of the total nitrogen (130), and the corresponding fraction from rabbit liver contained 15 per cent (79). A lower recovery of submicroscopic particles from mouse liver, reported by Barnum and Huseby (6), was later explained (66) on the basis of the use of isotonic NaCl as the medium, the electrolyte apparently having caused aggregation of the particles and difficulty in separating them from mitochondria (cf. [61]). The microsome content of rat kidney (132), on the basis of total nitrogen, was 16 per cent. The amount of nitrogen recovered in the submicroscopic particles of C3H mouse hepatoma 98/15 (130) was somewhat lower than the corresponding value obtained with normal C3H mouse liver. This finding is of some interest, since the mitochondrial content of the tumor was much lower than that of normal liver (130). Low, and approximately equal, amounts of nitrogen were recovered, however, in the mitochondria and microsomes of the Flexner-Jobling rat carcinoma (79).

One of the most striking properties of submicroscopic particles, first suggested by the experiments of Claude (23, 24), is a high concentration of PNA. In later studies (60, 125, 131, 132), it has been shown that approximately 50 per cent of the PNA of whole rat or mouse liver is present in this fraction. Furthermore, as indicated earlier in this review, microsomes comprise the only liver fraction in which PNA is concentrated in terms of total nitrogen (6, 55, 60, 66, 125, 131, 132), the average PNA/N ratio (micrograms PNA phosphorus per milligram nitrogen) being 63 (55, 60, 125, 132), and 64 (115, 131) for rat and mouse liver microsomes, as compared to 27 and 28 for the corresponding whole tissues. Although PNA was also found to be concentrated in the microsomes of rat kidney (132), the recovery of the nucleic acid in this fraction of kidney was considerably lower than that in the microsomes of liver. A high concentration of PNA was noted in the microsomes of hepatomas (115, 131) and a rat carcinoma (79) and to a lesser extent in the microsomes of both normal and leukemic spleen (3, 103). A decrease in the PNA content of liver microsomes of rats fed DAB and related compounds was noted by Price *et al.* (114, 116). Barnum and Huseby (7) have recently studied the relationship between the mammary tumor agent and the microsomes obtained from lactating mammary gland. It was found that a large percentage of microsome PNA could be removed without loss of mammary tumor agent activity. It was concluded that supposedly purified preparations of the agent consisted largely of microsomes.

Several studies have been made of the lipide content of liver submicroscopic particles (2, 6, 24, 66). The results are generally in agreement, in that a relatively high concentration of lipide, mostly in the form of phospholipide, is present in this fraction. The data indicate that about 40 per cent of the dry weight of the particles is composed of lipide, a value considerably higher than that reported for mitochondria. Some further information on the composition of the microsome fraction was presented by Barnum and Huseby (6), who separated the fraction by means of differential centrifugation into two components. The faster sedimenting microsomes were rich in both lipide and PNA, whereas the slower sedimenting particles were relatively poor in lipide but rich in PNA.

Another striking characteristic of the microsome fraction is the pronounced red color of the particles when packed by centrifugation. Bensley (10) succeeded in extracting this pigment and studied some of its properties. The conclusion was reached that the pigment represented products of the oxidation of unsaturated fats and particularly of phospholipides.

As mentioned previously, Lagerstedt (74) concluded that isolated microsomes are probably breakdown products of cytoplasmic basophilic inclusions. In a cytological study of the effect of fasting and protein depletion, Lagerstedt reported that under these conditions the basophilic inclusions completely disappeared. Some doubt as to the correctness of Lagerstedt's conclusion regarding the relation of microsomes and the inclusion bodies arises from the experiments of Muntwyler *et al.* (97). The latter investigators found that severe protein depletion resulted in some decline in the amount of submicroscopic particulate material but that the fraction by no means disappeared.

Relatively little is known concerning the enzymatic properties of the microsomes. The only

enzymes that have been shown thus far to occur in the fraction in a concentration exceeding that in whole tissue are an esterase (methyl butyrase) studied by Omachi *et al.* (99), DPN-cytochrome c reductase (55, 58), and TPN-cytochrome reductase (61). Heller and Bargoni (51) also studied the distribution of esterase in liver fractions and recovered most of the activity in a supernatant containing microsomes and soluble material. This supernatant was not further fractionated. The specific enzyme activities reported by the latter investigators were considerably lower than those reported by Omachi *et al.* (99). In this respect, in a recent study of the determination of esterase in homogenates, Copenhaver *et al.* (30) obtained specific activities similar to those reported by Omachi *et al.*

It may be noted that all three of the above enzymes are also present in the other particulate fractions of liver. Both esterase and DPN-cytochrome c reductase are concentrated to a much greater extent in the microsomes than in any other fraction; TPN-cytochrome c reductase, however, is slightly more concentrated in mitochondria than in microsomes. A relatively small proportion of the total ATP-ase activity of normal mouse liver is recovered in the submicroscopic particles (131). In the case of a mouse hepatoma, however, much more ATP-ase is recovered in this fraction (131). DPN-cytochrome c reductase is also more concentrated in hepatoma microsomes than in normal liver microsomes (58).

Submicroscopic particles also play a role in certain other enzyme reactions, such as anaerobic glycolysis (79), the oxidation of oxalacetate (132), and the reductive cleavage of DAB (94). In the case of the latter reaction, TPN was reduced by the final supernatant in the presence of glucose-6-phosphate, and the reduced TPN was utilized by the particulate fractions in cleaving DAB, the microsome fraction being more active than either nuclei or mitochondria. Tagnon and associates (138, 139) found that microsomes isolated from lung activated serum proplasmin. Claude (24) has stated that submicroscopic particles contain thromboplastic activity.

As mentioned previously, the question of the origin of the microsomes is at present unsettled. Recently (49, 50, 64), use has been made of the term microsomes, indicating that they may be products of disintegrated mitochondria. In this respect, although it has been shown that disintegration of mitochondria does yield particles of smaller size, it has also been clearly shown that the latter particles, in their content of respiratory enzymes (57), are qualitatively different from the microsomes isolated from liver homogenates by differential centrifugation.

Soluble or supernatant fraction.—This fraction contains the nonsedimentable or soluble material present in the cell, as well as any material, such as lipide droplets, which would migrate centripetally because of its low density. It is to be noted, however, that the preparation of this fraction is usually an arbitrary one dependent upon the limiting centrifugal force available to the investigator. The conditions that we have employed for the isolating of microsomes (129) were chosen to sediment particles as small as 50 mμ in diameter. Thus, the supernatant fluid or soluble fraction would contain all particles *smaller* than 50 mμ or, in terms of molecular weight, all particles having molecular weights less than about 100,000,000.

The soluble fraction comprises a considerable proportion of the tissue in terms of total nitrogen or protein. Thus, 29–42 per cent of the total nitrogen of mouse liver has been recovered in this fraction (6, 66, 130), while values of 32–44 per cent have been reported for rat liver (55, 60, 113, 125, 129, 132) and 49 per cent for rabbit liver (79). The proportions recovered in the supernatant fraction of rat kidney (132), primary rat liver tumors (115), mouse hepatoma (130), and Flexner-Jobling carcinoma (79) were 47, 46, and 50 per cent, respectively, while in the case of normal and leukemic mouse spleen considerably lower proportions were recovered in this fraction (27 and 23 per cent [103]).

PNA is also present in the soluble fraction, although in most tissues its concentration in this fraction is lower than in the whole tissue. However, in the case of normal and leukemic mouse spleen (103) and of rat kidney (132), the concentration of PNA in the supernatant is greater than in the whole tissue. Furthermore, the total amount of PNA in the soluble fraction of leukemic spleen is much greater than that in the same fraction obtained from normal spleen (103). Similar increases in the PNA content of the soluble fraction have been reported for primary rat liver tumors (115), mouse hepatoma (130), the livers of rats fed the carcinogen 3′-methyl-DAB (116), and regenerating rat liver (111). These findings, together with the observations of Brachet and Jeener (13, 67) on the high PNA content of the soluble fraction of rapidly growing cells suggest that the PNA associated with this fraction may be involved in the carcinogenic process or at least the process of growth.

The main component of the soluble fraction appears to be protein in nature. Thus, Price *et al.* (112) reported that 39 per cent of the total protein

of rat liver was recovered in the soluble fraction. A preliminary report of the electrophoretic properties of the soluble fraction of rabbit liver has been made by Sorof and Cohen (134), who found that four components were present. However, electrophoretic studies with calf thymus and human lymphoid tissue (1) indicate that the soluble fractions of these tissues were considerably more complex. Considering the enzymatic complexity of the supernatant it would appear unlikely that there could be as few as four components, unless the components were inhomogeneous or the enzymatic activities were bound to other proteins. Thus, in the case of rabbit liver and Flexner-Jobling carcinoma (79), all the enzymes involved in the glycolysis of glucose to lactic acid were found to be present in the soluble fraction, and the total activity of this fraction was sufficient to account for over 50 per cent of the total activity of the whole tissue. The mitochondria and microsomes of these tissues possessed essentially no glycolytic activity by themselves but produced pronounced stimulation of the supernatant, indicating that these fractions were deficient in some of the enzymes of the glycolytic cycle or that they contained necessary enzymes or cofactors required by the supernatant.

Other enzymes and related substances that occur in the supernatant of rat and mouse liver include cytochrome c (128, 129), isocitric dehydrogenase (61), and acid and alkaline phosphatase (98). The amount of cytochrome c present in the rat liver supernatant accounts for 35–40 per cent of the total liver cytochrome c, while in the case of isocitric dehydrogenase 82 per cent of the total liver activity was recovered in the supernatant. The distribution of acid and alkaline phosphatase in rat liver was considerably different from that of other phosphatases, such as ATP-ase (cf. above). Thus, in the case of the latter, most of the total liver activity was recovered in the nuclei and mitochondria, while 35–50 per cent of the total acid phosphatase and 55–70 per cent of the total alkaline phosphatase was recovered in the soluble fraction (98).

Miller and Miller (86) recently discovered that feeding azo dyes to rats resulted in the formation in the liver of dye-protein complexes in which the dye was tightly bound to the protein. The dye-protein complex was not found in other tissues of the rat, in the primary liver tumors produced by the dye, or in the tissues of other species in which these dyes are noncarcinogenic. The rate of formation of the dye-protein complexes in rat liver was found to be proportional to the carcinogenicity of the dye fed (87). In more recent studies (113–116), the intracellular distribution of the dye-protein has been studied, and it has been found that over 50 per cent of the protein-bound dye was always recovered in the supernatant fraction. The significance of this dye-protein complex and of its presence in the soluble fraction will require further study.

Julen, Snellman, and Sylven (69) have recently reported the results of cytological and fractionation studies made with mast cells in an attempt to determine the intracellular localization of heparin. The cytological studies indicated that the heparin was not bound to microscopically visible particles but was localized in the intergranular spaces. This was confirmed by fractionating mast cell extracts made by grinding ox liver capsules in a mortar with isotonic phosphate buffer and centrifuging at low speed to remove unbroken cells. The extract so obtained was further fractionated into large granules, microsomes, and a final supernatant remaining after 5–6 hours at 60,000 *g*. The latter contained 82 per cent of the heparin present in the cell-free extract. Electron microscopic examination of the supernatant showed the presence of particles with an estimated diameter less than 10 mμ. Electrophoretic and ultracentrifugal studies indicated that the heparin was bound to these particles in the form of a protein complex.

REFERENCES

1. Abrams, A., and Cohen, P. P. Electrophoretic and Chemical Characterization of Human Lymphoid Tissue and Calf Thymus. J. Biol. Chem., **177**:439–49, 1949.
2. Ada, G. L. Phospholipin Metabolism in Rabbit Liver Cytoplasm. Biochem. J., **45**:422–28, 1949.
3. Alfin-Slater, M. B.; Larack, A. M.; and Petermann, M. L. The Preparation and Properties of the Mitochondria and Submicroscopic Particles of Normal and of Leukemic Mouse Organs. Cancer Research, **9**:215–16, 1949.
4. Arnesen, K.; Goldsmith, Y.; and Dulaney, A. D. Antigenic Properties of Nuclei Segregated from Spleens of Normal and Leukemic Mice. Cancer Research, **9**:669–71, 1949.
5. Baker, C. G., and Meister, A. Studies on Fatty Acid Oxidation by Normal and Neoplastic Liver. J. Nat. Cancer Inst., **10**:1191–98, 1950.
6. Barnum, C. P., and Huseby, R. A. Some Quantitative Analyses of the Particulate Fractions from Mouse Liver Cytoplasm. Arch. Biochem., **19**:17–23, 1948.
7. ———. The Chemical and Physical Characteristics of Preparations Containing the Milk Agent Virus: *A Review*. Cancer Research, **10**:523–29, 1950.
8. Barnum, C. P.; Nash, C. W.; Jennings, E.; Nygaard, O.; and Vermund, H. The Separation of Pentose and Desoxypentose Nucleic Acids from Isolated Mouse Liver Cell Nuclei. Arch. Biochem., **25**:376–83, 1950.
9. Behrens, M. Untersuchungen an isolierten Zell- und Gewebsbestandteilen. I. Isolierung von Zellkernen des Kalbsherzmuskels. Ztschr. f. physiol. Chem., **209**:59, 1932.

10. BENSLEY, R. R. On the Nature of the Pigment of Mitochondria and of Submicroscopic Particles in the Hepatic Cell of the Guinea Pig. Anat. Rec., **98**:609–20, 1947.
11. BENSLEY, R. R., and HOERR, N. The Preparation and Properties of Mitochondria. Anat. Rec., **60**:449–55, 1934.
12. BORSOOK, H. Protein Turnover and Incorporation of Labeled Amino Acids into Tissue Proteins *in vivo* and *in vitro*. Physiol. Rev., **30**:206–19, 1950.
13. BRACHET, J., and JEENER, R. Recherches sur des particules cytoplasmiques de dimensions macromoléculaires riches en acide pentosenucléique. I. Propriétés générales, relations avec les hormones, les protéines de structure. Enzymologia, **11**:196–212, 1944.
14. BRADFIELD, J. R. G. The Localization of Enzymes in Cells. Biol. Rev. (Cambridge), **25**:113–57, 1950.
15. BRENNER, S. The Demonstration by Supravital Dyes of Oxidation-Reduction Systems on the Mitochondria of the Intact Rat Lymphocyte. S. Afr. J. M. Sc., **14**:13–19, 1949.
16. CHAMBERS, R. The Isolation of Visible Protoplasmic Structures. Cancer Research, **10**:210, 1950.
17. CHANTRENNE, H. Recherches sur des particules cytoplasmiques de dimensions macromoléculaires riches en acide pentosenucléique. II. Relations avec les ferments respiratoires. Enzymologia, **11**:213–21, 1944.
18. ———. Hétérogénéité des granules cytoplasmiques du foie de souris. Biochem et Biophys. Acta, **1**:437–48, 1947.
19. CLAUDE, A. Particulate Components of Cytoplasm. Cold Spring Harbor Symp. Quant. Biol., **9**:263–70, 1941.
20. ———. The Constitution of Protoplasm. Science, **97**: 451–56, 1943.
21. ———. Distribution of Nucleic Acids in the Cell and the Morphological Constitution of Cytoplasm. Biol. Symp., **10**:111–29, 1943.
22. ———. Distribution of Enzymatic Activities in Fractions of Mammalian Liver. A.A.A.S. Research Conference on Cancer, pp. 223–26, 1944.
23. ———. The Constitution of Mitochondria and Microsomes and the Distribution of Nucleic Acid in the Cytoplasm of a Leukemic Cell. J. Exper. Med., **80**:19–29, 1944.
24. ———. Fractionation of Mammalian Liver Cells by Differential Centrifugation. I. Problems, Method, and Preparation of Extract. II. Experimental Procedures and Results. *Ibid.*, **84**:51–98, 1946.
25. ———. Studies on Cells: Morphology, Chemical Constitution, and Distribution of Biochemical Functions. Harvey Lectures, **43**:121–64, 1947–48.
26. CLAUDE, A., and FULLAM, E. F. An Electron Microscope Study of Isolated Mitochondria. J. Exper. Med., **81**: 51–62, 1945.
27. ———. The Preparation of Sections of Guinea Pig Liver for Electron Microscopy. *Ibid.*, **83**:499–504, 1946.
28. CLAUDE, A., and POTTER, J. S. Isolation of Chromatin Threads from the Resting Nucleus of Leukemic Cells. J. Exper. Med., **77**:345–54, 1943.
29. COHEN, P. P., and MCGILVERY, R. W. Peptide Bond Synthesis. III. On the Mechanism of *p*-Aminohippuric Acid Synthesis. J. Biol. Chem., **171**:121–33, 1947.
30. COPENHAVER, J. H.; STAFFORD, R. O.; and MCSHAN, W. H. The Determination of Esterase in Animal Tissue Homogenates. Arch. Biochem., **26**:260–68, 1950.
31. CUNNINGHAM, L.; GRIFFIN, A. C.; and LUCK, J. M. Effect of a Carcinogenic Azo Dye on Liver Cell Structure. Isolation of Nuclei and Cytoplasmic Granules. Cancer Research, **10**:194–99, 1950.
32. DALTON, A. J.; KAHLER, H.; KELLY, M. G.; LLOYD, B. J.; and STRIEBICH, M. J. Some Observations on the Mitochondria of Normal and Neoplastic Cells with the Electron Microscope. J. Nat. Cancer Inst., **9**:439–49, 1949.
33. DANIELLI, J. F. Establishment of Cytochemical Techniques. Nature, **157**:755–57, 1946.
34. DOUNCE, A. L. Enzyme Studies on Isolated Cell Nuclei of Rat Liver. J. Biol. Chem., **147**:685–98, 1943.
35. ———. Further Studies on Isolated Cell Nuclei of Normal Rat Liver. *Ibid.*, **151**:221–33, 1943.
36. ———. Enzyme Systems of Isolated Cell Nuclei. Ann. N.Y. Acad. Sc., **50**:982–99, 1950.
37. DOUNCE, A. L.; TISHKOFF, G. H.; BARNETT, S. R.; and FREER, R. M. Free Amino Acids and Nucleic Acid Content of Cell Nuclei Isolated by a Modification of the Behrens' Technique. J. Gen. Physiol., **33**:629–42, 1950.
38. DUBUY, H. G.; WOODS, M. W.; BURK, D.; and LACKEY, M. D. Enzymatic Activities of Isolated Amelanotic and Melanotic Granules of Mouse Melanomas and a Suggested Relationship to Mitochondria. J. Nat. Cancer Inst., **9**:325–36, 1949.
39. DUBUY, H. G.; WOODS, M. W.; and LACKEY, M. D. Enzymatic Activities of Isolated Normal and Mutant Mitochondria and Plastids of Higher Plants. Science, **111**: 572–74, 1950.
40. DULANEY, A. D.; GOLDSMITH, Y.; ARNESEN, K.; and BUXTON, L. A Serological Study of Cytoplasmic Fractions from the Spleens of Normal and Leukemic Mice. Cancer Research, **9**:217–21, 1949.
41. ERNSTER, L.; ZETTERSTRÖM, R.; and LINDBERG, O. Vitamin A as a Component in the Mechanism of Aerobic Energy Transport. Exper. Cell. Research, **1**:494–96, 1950.
42. EULER, H. VON, and HELLER, L. Katalaseaktivität in Leberfraktionen normaler und sarkomtragender Ratten. Ztschr. Krebsforsch., **56**:393–406, 1949.
43 GOERNER, A., and GOERNER, M. M. Vitamin A and Tumor Mitochondria. J. Biol. Chem., **123**:57–59, 1938.
44. GOMORI, G. The Microchemical Demonstration of Sites of Lipase Activity. Proc. Soc. Exper. Biol. & Med., **58**: 362–64, 1945.
45. GOPAL-AYENGAR, A. R., and COWDRY, E. V. Desoxyribose Nucleic Acid from Isolated Chromosome Threads in Experimental Epidermal Methylcholanthrene Carcinogenesis in Mice. Cancer Research, **7**:1–8, 1947.
46. GRAFFI, A., and JUNKMAN, K. Beitrag zum chemischen Aufbau normaler und maligner Zellen. Klin. Wchnschr., **24**:78–81, 1946.
47. GREEN, D. E.; LOOMIS, W. F.; and AUERBACH, V. H. Studies on the Cyclophorase System. I. The Complete Oxidation of Pyruvic Acid to Carbon Dioxide and Water. J. Biol. Chem., **172**:389–403, 1948.
48. GRIFFIN, A. C.; COOK, H.; and CUNNINGHAM, L. Tissue Proteins and Carcinogenesis. III. Precancerous Changes in the Liver and Serum Protein of Rats Fed Acetylaminofluorene. Arch. Biochem., **24**:190–98, 1949.
49. HARMAN, J. W. Studies on Mitochondria. I. The Association of Mitochondria with Cyclophorase. Exper. Cell. Research, **1**:382–93, 1950.
50. ———. Studies on Mitochondria. II. The Structure of Mitochondria in Relationship to Enzymatic Activity. *Ibid.*, pp. 394–402, 1950.
51. HELLER, L., and BORGONI, N. Studien über die intrazellulare Verteilung der Enzyme. III. Die intrazellulare Verteilung der Monobutyrase in der Leber normaler und sarkomatöser Ratten. Arkiv Kemi, **1**:447–54, 1950.
52. HERMANN, H., and BOSS, M. B. Dopa Oxidase Activity in Extracts from Ciliary Body and in Isolated Pigment Granules. J. Cell. & Comp. Physiol., **26**:131–38, 1945.
53. HIRD, F. J. R., and ROWSELL, E. V. Additional Trans-

amination by Insoluble Particle Preparations of Rat Liver. Nature, **166**:517–18, 1950.

54. Hoerr, N. L. Methods of Isolation of Morphological Constituents of the Liver Cell. Biol. Symp., **10**:185–231, 1943.
55. Hogeboom, G. H. Cytochemical Studies of Mammalian Tissues. II. The Distribution of Diphosphopyridine-nucleotide-Cytochrome c Reductase in Rat Liver Fractions. J. Biol. Chem., **177**:847–58, 1949.
56. Hogeboom, G. H.; Claude, A.; and Hotchkiss, R. D. The Distribution of Cytochrome Oxidase and Succinoxidase in the Cytoplasm of the Mammalian Liver Cell. J. Biol. Chem., **165**:615–29, 1946.
57. Hogeboom, G. H., and Schneider, W. C. Sonic Disintegration of Isolated Liver Mitochondria. Nature, **166**: 302–303, 1950.
58. ———. Intracellular Distribution of Enzymes. VIII. The Distribution of Diphosphopyridinenucleotide-Cytochrome c Reductase in Normal Mouse Liver and Mouse Hepatoma. J. Nat. Cancer Inst., **10**:983–87, 1950.
59. ———. Proteins of Liver and Hepatoma Mitochondria. Science (in press).
60. Hogeboom, G. H.; Schneider, W. C.; and Pallade, G. E. Cytochemical Studies of Mammalian Tissues. I. Isolation of Intact Mitochondria from Rat Liver; Some Biochemical Properties of Mitochondria and Submicroscopic Particulate Material. J. Biol. Chem., **172**:619–35, 1948.
61. Hogeboom, G. H., and Schneider, W. C. Cytochemical Studies of Mammalian Tissues. III. Isocitric Dehydrogenase and Triphosphopyridine Nucleotide-Cytochrome c Reductase in Mouse Liver. J. Biol. Chem., **186**:417–27, 1950.
62. Holter, H. Establishment of Cytochemical Techniques. Nature, **158**:917, 1946.
63. Hoster, M. S.; McBee, B. J.; Rolnick, H. A.; van Winkle, Q.; and Hoster, H. A. Macromolecular Particles Obtained from Human Neoplastic and Non-neoplastic Lymph Nodes. I. Procedure and Preliminary Results. Cancer Research, **10**:530–38, 1950.
64. Huennekens, F. M., and Green, D. E. Studies on the Cyclophorase System. X. The Requirement for Pyridine Nucleotide. Arch. Biochem., **27**:418–27, 1950.
65. ———. Studies on the Cyclophorase System. XI. The Effect of Various Treatments on the Requirement for Pyridine Nucleotide. *Ibid.*, pp. 428–40, 1950.
66. Huseby, R. A., and Barnum, C. P. Investigation of the Phosphorus-containing Constituents of Centrifugally Prepared Fractions from Mouse Liver Cell Cytoplasm. Arch. Biochem., **26**:187–98, 1950.
67. Jeener, R., and Brachet, J. Recherches sur l'acide ribonucléique des levures. Enzymologia, **11**:222–34, 1944.
68. Jeener, R., and Szaforz, D. Relation between the Rate of Renewal and the Intracellular Localization of Ribonucleic Acid. Arch. Biochem., **26**:54–67, 1950.
69. Julen, C.; Snellman, O.; and Sylven, B. Cytological and Fractionation Studies on the Cytoplasmic Constituents of Tissue Mast Cells. Acta Physiol. Scandinav., **19**:289–305, 1950.
70. Kennedy, E. P., and Lehninger, A. L. Intracellular Structures and the Fatty Acid Oxidase System of Rat Liver. J. Biol. Chem., **172**:847–48, 1948.
71. ———. Oxidation of Fatty Acids and Tricarboxylic Acid Cycle Intermediates by Isolated Rat Liver Mitochondria. *Ibid.*, **179**:957–72, 1949.
72. Kielley, R. K., and Schneider, W. C. Synthesis of *p*-Aminohippuric Acid by Mitochondria of Mouse Liver Homogenates. J. Biol. Chem., **185**:869–80, 1950.
73. Kurnick, N. B. The Quantitative Estimation of Desoxyribose Nucleic Acid Based on Methyl Green Staining. Exper. Cell. Research, **1**:151–58, 1950.
74. Lagerstedt, S. Cytological Studies on the Protein Metabolism of the Liver in Rat. Acta Anatomica, Suppl. IX, 1949.
75. Lambden, M. P. Considerations on Indiscriminate Use of the Waring Blendor: Copper Contamination and Ascorbic Acid Loss. Fed. Proc., **9**:193, 1950.
76. Lazarow, A. Particulate Glycogen: A Submicroscopic Component of the Guinea Pig Liver Cell; Its Significance in Glycogen Storage and the Regulation of Blood Sugar. Anat. Rec., **84**:31–50, 1942.
77. ———. The Chemical Structure of Cytoplasm as Investigated in Professor Bensley's Laboratory during the Past 10 Years. Biol. Symp., **10**:9–26, 1943.
78. Lehninger, A. L., and Kennedy, E. P. The Requirements of the Fatty Acid Oxidase Complex of Rat Liver. J. Biol. Chem., **173**:753–71, 1948.
79. LePage, G. A., and Schneider, W. C. Centrifugal Fractionation of Glycolytic Enzymes in Tissue Homogenates. J. Biol. Chem., **176**:1021–27, 1948.
80. Leuthardt, F., and Müller, A. F. Mitochondrien und Citrullensynthese in der Leber. Experientia, **4**:478, 1948.
81. Li, C., and Roberts, E. Amino Acids in the Mitochondrial Fractions of Tissues as Determined by Paper Partition Chromatography. Science, **110**:559–60, 1949.
82. Linderstrom-Lang, K. Distribution of Enzymes in Tissues and Cells. Harvey Lectures, **34**:214–45, 1938–39.
83. Marshak, A. P^{32} Uptake by Nuclei. J. Gen. Physiol., **25**:275–91, 1941.
84. Marshak, A., and Calvet, F. Specific Activity of P^{32} in Cell Constituents of Rabbit Liver. J. Cell. & Comp. Physiol., **34**:451–56, 1949.
85. Mayer, D. T., and Gulick, A. The Nature of the Proteins of Cellular Nuclei. J. Biol. Chem., **146**:433–40, 1942.
86. Miller, E. C., and Miller, J. A. The Presence and Significance of Bound Amino-Azo Dyes in the Livers of Rats Fed *p*-Dimethyl Aminoazobenzene. Cancer Research, **7**:468–80, 1947.
87. Miller, E. C.; Miller, J. A.; Sapp, R. W.; and Weber, G. M. Studies on the Protein-bound Amino-Azo Dyes Formed *in vivo* from 4-Dimethylamino-azobenzene and Its c-Monomethyl Derivatives. Cancer Research, **9**:336–43, 1949.
88. Milner, H. W.; Lawrence, N. S.; and French, C. S. Colloidal Dispersion of Chloroplast Material. Science, **111**:633–34, 1950.
89. Mirsky, A. E. Chemical Properties of Isolated Chromosomes. Cold Spring Harbor Symp. Quant. Biol., **12**:143–46, 1947.
90. Mirsky, A. E., and Pollister, A. W. Fibrous Nucleoproteins of Chromatin. Biol. Symp., **10**:247–60, 1943.
91. Mirsky, A. E., and Ris, H. Isolated Chromosomes. J. Gen. Physiol., **31**:1–6, 1947.
92. ———. The Chemical Composition of Isolated Chromosomes. *Ibid.*, pp. 7–18, 1947.
93. ———. Variable and Constant Components of Chromosomes. Nature, **163**:666–67, 1949.
94. Mueller, G. C., and Miller, J. A. The Reductive Cleavage of 4-Dimethylamino-azobenzene by Rat Liver: The Intracellular Distribution of the Enzyme System and Its Requirement for Triphosphopyridine Nucleotide. J. Biol. Chem., **180**:1125–36, 1949.
95. Mühlethaler, K.; Müller, A. F.; and Zollinger, H. U. Zur Morphologie der Mitochondrien. Experientia, **6**:16, 1950.
96. Müller, A. F., and Leuthardt, F. Oxydative Phos-

phorylierung und Citrullinsynthese in den Leber Mitochondrien. Helvet. chem. acta, **32**:2349–56, 1949.

97. MUNTWYLER, E.: SEIFTER, S.; and HARKNESS, D. M. Some Effects of Restriction of Dietary Protein on the Intracellular Components of Liver. J. Biol. Chem., **184**: 181–90, 1950.
98. NOVIKOFF, A. B.; PODBER, E.; and RYAN, J. Intracellular Distribution of Phosphatase Activity in Rat Liver. Fed. Proc., **9**:210, 1950.
99. OMACHI, A.; BARNUM, C. P.; and GLICK, D. Quantitative Distribution of an Esterase among Cytoplasmic Components of Mouse Liver Cells. Proc. Soc. Exper. Biol. & Med., **67**:133–36, 1948.
100. OPIE, E. L. The Movement of Water in Tissues Removed from the Body and Its Relation to Movement of Water during Life. J. Exper. Med., **89**:185–208, 1949.
101. PALADE, G. E., and CLAUDE, A. The Nature of the Golgi Apparatus. I. Parallelism between Golgi Apparatus and Intracellular Myelin Figures. J. Morphol., **85**:35–70, 1949.
102. ———. The Nature of the Golgi Apparatus. II. Identification of the Golgi Apparatus with a Complex of Myelin Figures. *Ibid.*, pp. 71–112, 1949.
103. PETERMANN, M. L.; ALFIN-SLATER, R. B.; and LARACK, A. M. The Nucleic Acid Distribution in Normal and Leukemic Mouse Spleen. Cancer, **2**:510–15, 1949.
104. PETERMANN, M. L., and MASON, E. J. Nucleic Acid Content of Chromosomes of Normal and Leukemic Mouse Spleen. Proc. Soc. Exper. Biol. & Med., **69**:542–44, 1948.
105. POLLISTER, A. W., and LEUCHTENBERGER, C. The Nucleoprotein Content of Whole Nuclei. Proc. Nat. Acad. Sc., **35**:66–71, 1949.
106. PORTER, K. R.; CLAUDE, A.; and FULLAM, E. F. A Study of Tissue Culture Cells by Electron Microscopy. J. Exper. Med., **81**:233–46, 1945.
107. POTTER, V. R., and ELVEHJEM, C. A. A Modified Method for the Study of Tissue Oxidations. J. Biol. Chem., **114**: 495–504, 1936.
108. POTTER, V. R., and LE PAGE, G. A. Metabolism of Oxalacetate in Glycolyzing Tumor Homogenates. J. Biol. Chem., **177**:237–45, 1949.
109. POTTER, V. R.; PRICE, J. M.; MILLER, E. C.; and MILLER, J. A. Studies on the Intracellular Composition of Livers from Rats Fed Various Aminoazo Dyes. III. Effects on Succinoxidase and Oxalacetic Acid Oxidase. Cancer Research, **10**:28–35, 1950.
110. POTTER, V. R., and SCHNEIDER, W. C. Oxidative Phosphorylation without Deamination in Mitochondrial Preparations. Fed Proc., **8**:237, 1949.
111. PRICE, J. M., and LAIRD, A. K. A Comparison of the Intracellular Composition of Regenerating Liver and Induced Liver Tumors. Cancer Research, **10**:650–58, 1950.
112. PRICE, J. M.; MILLER, E. C.; and MILLER, J. A. The Intracellular Distribution of Protein, Nucleic Acids, Riboflavin, and Protein-bound Aminoazo Dye in the Livers of Rats Fed *p*-Dimethylaminoazobenzene. J. Biol. Chem., **173**:345–53, 1948.
113. ———. Intracellular Distribution of Vitamin B_6 in Rat and Mouse Livers and Induced Rat Liver Tumors. Proc. Soc. Exper. Biol. & Med., **71**:575–78.
114. PRICE, J. M.; MILLER, E. C; MILLER, J. A.; and WEBER, G. M. Studies on the Intracellular Composition of Livers from Rats Fed Various Aminoazo Dyes. I. Cancer Research, **9**:398–402, 1949.
115. ———. Studies on the Intracellular Composition of Liver and Liver Tumor from Rats Fed 4-Dimethylaminoazobenzene. *Ibid.*, pp. 96–102.
116. ———. Studies on the Intracellular Composition of Liver from Rats Fed Various Aminoazo Dyes. II. 3′Methyl-, 2′ Methyl-, and 2 Methyl-4-Dimethylaminoazobenzene, 3-Methyl-4-Dimethylaminoazobenzene, and 4′-Fluoro-4-Dimethylaminoazobenzene. *Ibid.*, **10**:18–27, 1950.
117. RECKNAGEL, R. O. Localization of Cytochrome Oxidase on the Mitochondria of the Frog Egg. J. Cell. & Comp. Physiol., **35**:111–30, 1950.
118. RIS, H., and MIRSKY, A. E. The State of the Chromosomes in the Interphase Nucleus. J. Gen. Physiol., **32**: 489–502, 1949.
119. ———. Quantitative Cytochemical Determination of Desoxyribosenucleic Acid with the Feulgen Nucleal Reaction. *Ibid.*, **33**:125–45, 1950.
120. SCHEIN, A. H.; PODBER, E.; and NOVIKOFF, A. B. Intracellular Localization of Uricase Activity. Fed. Proc., **9**: 224, 1950.
121. SCHNEIDER, W. C. Phosphorus Compounds in Animal Tissues. II. The Nucleic Acid Content of Homologous Normal and Cancer Tissues. Cancer Research, **5**:717–21, 1945.
122. ———. Intracellular Distribution of Enzymes. I. The Distribution of Succinic Dehydrogenase, Cytochrome Oxidase, Adenosinetriphosphatase, and Phosphorus Compounds in Normal Rat Tissues. J. Biol. Chem., **165**:585–93, 1946.
123. ———. Intracellular Distribution of Enzymes. II. The Distribution of Succinic Dehydrogenase, Cytochrome Oxidase, Adenosinetriphosphatase and Phosphorus Compounds in Normal Rat Liver and in Rat Hepatomas. Cancer Research, **6**:685–90, 1946.
124. ———. Nucleic Acids in Normal and Neoplastic Tissues. Cold Spring Harbor Symp. Quant. Biol., **12**:169–77, 1947.
125. ———. Intracellular Distribution of Enzymes. III. The Oxidation of Octanoic Acid by Rat Liver Fractions. J. Biol. Chem., **176**:259–66, 1948.
126. ———. The Distribution of Enzymes within the Cell, p. 273. In: Respiratory Enzymes. Minneapolis: Burgess Publishing Co., 1949.
127. ———. Methods for the Isolation of Particulate Components of the Cell, p. 148. In: Manometric Techniques and Tissue Metabolism. Minneapolis: Burgess Publishing Co., 1949.
128. SCHNEIDER, W. C.; CLAUDE, A.; and HOGEBOOM, G. H. The Distribution of Cytochrome c and Succinoxidase Activity in Rat Liver Fractions. J. Biol. Chem., **172**: 451–58, 1948.
129. SCHNEIDER, W. C., and HOGEBOOM, G. H. Intracellular Distribution of Enzymes. V. Further Studies on the Distribution of Cytochrome c in Rat Liver Homogenates. J. Biol. Chem., **183**:123–28, 1950.
130. ———. Intracellular Distribution of Enzymes. IV. The Distribution of Succinoxidase and Cytochrome Oxidase Activities in Normal Mouse Liver and in Mouse Hepatoma. J. Nat. Cancer Inst., **10**:969–75, 1950.
131. SCHNEIDER, W. C.; HOGEBOOM, G. H.; and ROSS, H. E. Intracellular Distribution of Enzymes. VII. The Distribution of Nucleic Acids and Adenosinetriphosphatase in Normal Mouse Liver and Mouse Hepatoma. J. Nat. Cancer Inst., **10**:977–82, 1950.
132. SCHNEIDER, W. C., and POTTER, V. R. Intracellular Distribution of Enzymes. IV. The Distribution of Oxalacetic Oxidase Activity in Rat Liver and Rat Kidney Fractions. J. Biol. Chem., **177**:893–903, 1949.
133. SCHWEIGERT, B. S.; GUTHNECK, B. T.; PRICE, J. M.; MILLER, J. A.; and MILLER, E. C. Amino Acid Composition of Morphological Fractions of Rat Livers and

Induced Liver Tumors. Proc. Soc. Exper. Biol. & Med., **72**:495–501, 1949.

134. Sorof, S., and Cohen, P. P. Electrophoretic Studies of the Solubie Protein of Rabbit Liver. Fed. Proc., **8**:254, 1949.
135. Steinbach, H. B., and Moog, F. The Localization of Adenylpyrophosphatase in Cytoplasmic Granules. J. Cell. & Comp. Physiol., **26**:175–83, 1945.
136. Still, J. E., and Kaplan, E. H. Localization of Oxidases. Exper. Cell. Research, **1**:403–9, 1950.
137. Swift, H. H. The Desoxyribose Nucleic Acid Content of Animal Nuclei. Physiol. Zoöl., **23**:169–98, 1950.
138. Tagnon, H. J., and Palade, G. E. Activation of protoplasmin by a Factor from Mammalian Tissue. J. Clin. Investigation, **29**:317–24, 1950.
139. Tagnon, H. J., and Petermann, M. L. Activation of Proplasmin by a Tissue Fraction. Proc. Soc. Exper. Biol. & Med., **70**:359–60, 1949.
140. Tipton, S. R.; Leath, M. J.; Tipton, I. H.; and Nixon, W. L. The Effects of Feeding Thyroid Substance and of Adrenalectomy on the Activities of Succinoxidase and Cytochrome Oxidase in the Liver Tissue of Rats. Am. J. Physiol., **145**:693–98, 1946.
141. Wilson, E. B. The Cell in Development and Heredity, p. 85. New York: Macmillan Co., 1928.
142. Woods, M. W.; DuBuy, H. B.; Burk, D.; and Hesselbach, M. L. Cytological Studies on the Nature of the Cytoplasmic Particulates in the Cloudman S 91 Mouse Melanoma, the Derived Algire S 91A Partially Amelanotic Melanoma and the Harding-Passey Mouse Melanoma. J. Nat. Cancer Inst., **9**:311–24, 1949.
143. Zollinger, H. U. Cytologic Studies with the Phase Microscope. II. The Mitochondria and Other Cytoplasmic Constituents under Various Experimental Conditions. Am. J. Path., **24**:569–88, 1948.
144. ———. Zur qualitativen Nucleoproteingehalt und zur Morphologie der Mitochondrien. Experientia, **6**:14–19, 1950.

The Changes in Cell Morphology and Histochemistry of the Testis Following Irradiation and Their Relation to Other Induced Testicular Changes*

I. Quantitative Random Sampling of Germinal Cells at Intervals following Direct Irradiation

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The investigations reported here are directed primarily at the fundamental problems of growth, regeneration, and the differential response of various normal tissue elements to changes induced by x-radiation in the testes of adult pure-strain mice. These data are considered to contribute basic information concerning neoplasia, inasmuch as they deal with quantitative measurements of the fundamental phenomena of growth and regeneration, the normal range of regenerative growth, and the differential response of various tissue elements. We view the use of ionizing radiation as a tool for the study of growth.

A review of the literature shows that considerable material has been published regarding the effects of radiation on the testis. Among these are the results reported by Warren (14), Lea (8), Bloom (1), and Eschenbrenner and Miller (3). Most of these authors presented conclusions based on many kinds of ionizing radiation on various animals with a variety of radiation factors and doses.

There is agreement among all the authors with regard to (*a*) the gonia being the most sensitive of the germinal cells and (*b*) the interstitial cells being resistant to the minimum dose which will affect the germinal cells.

Bloom (1) is in variance with the general belief that mitosis is a predisposing factor toward radiation damage in the testis. He points out that the meiotic cytes are relatively radio-resistant when in complex stages of division, and he recommended further study of this observation.

Eschenbrenner and Miller (3) suggest that all stages of spermatogenesis are equally sensitive to radiation and that possibly chronic over-all irradiation would result in a retardation of the rate of production of gonia. They also suggest that the decrease of spermatozoa and the degeneration of the spermatids are not due directly to radiation, but in part, at least, to degeneration of Sertoli cells.

Liebow, Warren, and DeCoursey (10) suggest that the germinal epithelium or its derivatives may have an endocrine function. They base this suggestion on the reported appearance of "castration cells" in the pituitary body after irradiation of the testes (Joel, [7]). Castration cells may also appear in the pituitary body after ligation of the ductuli efferentes as reported by Van Wagener (13). Howard, Sniffen, Simmons, and Albright (5) suggest the possibility that germinal cells secrete a hormone and base their opinion on the presence of the castration cells. LeRoy (9), in his report on the medical sequelae from cases resulting from the atomic bomb explosions in Nagasaki and Hiroshima, describes complete destruction of the germinal cells with preservation of the Sertoli cells and also reports the presence of castration cells in the pituitary.

Tullis and Barrow (12) report a difference in sensitivity between what they call prespermatogonia and spermatogonia, but the spermatocytes were not examined from the point of view of comparative sensitivity.

It is quite evident from the diverse opinions in the literature that there are many problems un-

* This work was supported by Grant C-1047 from the Public Health Service and by the Department of Public Health, Pondville Hospital.

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Received for publication July 7, 1950.

solved regarding the germinal and endocrine relationship following radiation. It has not been clearly demonstrated whether cells other than gonia can give rise to germinal cells. The time necessary for the development from gonia to sperm in the adult animal has not been definitely determined.

One of the chief difficulties in evaluating the observed alterations following irradiation of testes has been the lack of technic for measurement of normal biological variation, particularly with respect to proliferation of germ cells. The recently reported Chalkley (2) method, however, is conclusive in determining the frequency of appearance of any given cell. The first aim, therefore, was to establish a norm against which cellular alterations due to irradiation might be compared, and the second was to choose a method of statistical analysis for numerical estimation and evaluation of radiation effects. This has been done by taking a quantitative random sampling of the germinal elements of the testis plotted as frequency of cell population types.

METHODS OF PROCEDURE AND OBSERVATIONS

The testes of 104 young, adult pure line C57 black mice, weighing 18–25 gm., were subjected to a single exposure of 300 r with the following factors: 100 KvP,[1] 15 Ma, 2.6 mm. Al, Hvl,[2] 187 r/minute at TSD[3] of 20 cm.

The method of irradiation was as follows: After strapping the animals to thin strips of wood, the strips were set in a holder at an angle of 45°, shielded with $\frac{1}{16}$ inch of lead, except for a small portal through which the testes were drawn. In this way, the testes and distal end of the colon were the only parts exposed.

Following radiation two mice were killed at 4 hours, and on each of the days indicated in Charts 1–4, with the following exceptions: ten mice were killed on days 4, 6, 7, 8, 12, 18, 21, and 26. Final observations, not indicated on the charts, were made at 8 and 12 weeks post-radiation. The ten animals killed at selected intervals were utilized to check possible biological variation from animal to animal.

Both testes were removed and placed in Zenkers solution for fixation; representative serial sections were then cut and stained with hematoxylin and eosin.

In another series of experiments, 100 animals were immobilized and given a single total-body exposure of 300 r without shielding. These animals were killed at the same intervals as above, up to 8 weeks, and preparations of the testes were made as in the foregoing group.

There were two classes of controls. In the first, the animals were not immobilized nor radiated. In the second, ten animals were subjected to the same immobilization procedure but were not radiated. Preparations and observations of these were followed through for 3 weeks.

The procedure for determining the incidence of germinal cells was as follows: slides representative of the whole of both testes were studied at a magnification of ×430, and from these slides 100 fields were studied; each field is not a microscopic field but a carefully selected median cross section of a tubule. The incidence of the germinal cell types (spermatogonia, spermatocytes, spermatids, and sperm) in these median cross sections at various periods post-radiation, as indicated in Charts 1–4, is the basis of this method of sampling. The number of times that any specific type of germinal cell appeared in 100 fields was approximately the same for the right and left testes of any animal, and, because of this, the counts on both testes were considered as a unit.

Ten untreated animals were used as the first set of controls. The pair of testes showing the highest incidence of spermatogonia revealed them in 87 of 100 fields. The testes with the lowest incidence revealed spermatogonia in 74 of 100 fields. Averaging the tabulated counts from the testes of ten animals gave a figure of 80. The same procedure was used for determining the average for each type of germinal cell, and the following results were obtained: 82 for spermatocytes, 88 for spermatids, and 85 for sperm. It is to be noted that control animals showed essentially the same average incidence for all types of the germinal cells.

It should be emphasized that this method of evaluation does not provide a qualitative analysis, nor is it strictly quantitative. It is possible that effects of radiation may have irreparably damaged some cells, but, at the time of counting, they had not lost their morphologic identity. For example, apparently normal sperm may be incapable of fertilization. The germ cells may be numerous or scarce in a field, but no attempt has been made to count them. The method of tabulation deals only with the presence or absence of selected germ cells in 100 selected median cross sections of the tubules.

At the 1-day interval after irradiation, only 42 of the fields revealed spermatogonia on the average; 90 showed spermatocytes, 92 showed spermatids, and 83 showed sperm. In the same manner, 5–7 days after irradiation (on the average for

[1] Peak kilovoltage.

[2] Half value layer.

[3] Target Skin Distance.

ten animals), only 10–11 fields will reveal spermatogonia. In 35 days, the average numerical incidence of germinal elements per 100 fields is approaching normal. The period of minimal average incidence of appearance for spermatocytes is not reached until after the fourteenth day, which is approximately 7 days later than the minimal average incidence for spermatogonia. The spermatids and sperm are at their minimal frequency at approximately 28 days, 14 days later than the spermatogonia. The sperm are the last to show reduced incidence of appearance (see Charts 1–4).

Complete disappearance of any of the germinal elements was not caused by direct exposure of the testes to 300 r.

The spermatocytes did not show a marked decrease in frequency until after the seventh day. Once decrease was apparent, it took approximately 7 days for them to reach their minimal frequency. In this respect they reacted the same as the spermatogonia, but the period of recovery was longer for the spermatogonia.

A similar delayed reaction to the effects of radiation was noted for the spermatids and sperm. The spermatids prevailed in normal numerical frequency until after the twelfth day, which was approximately 7 days later than the spermatocytes. The sperm were at normal numerical frequency for 21 days. By 12 weeks (not shown in the figures) the frequency for spermatocytes, spermatids, and

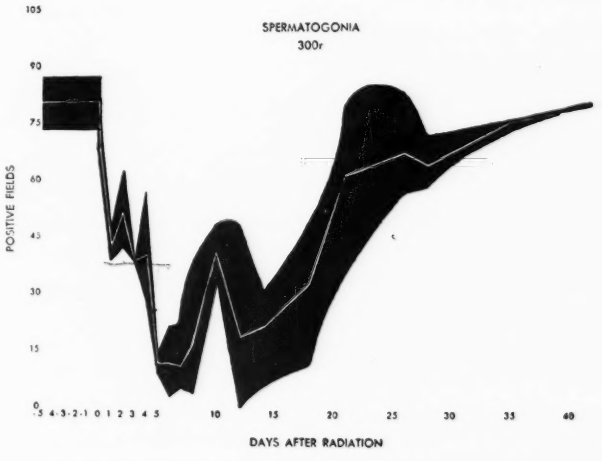

CHART 1.—Numerical frequency of spermatogonia plotted against time following direct radiation of testis, 300 r. The white line represents the average frequency calculated from the frequency determined for each animal used at any given interval. The black represents the spread of points obtained from animals studied at a given interval.

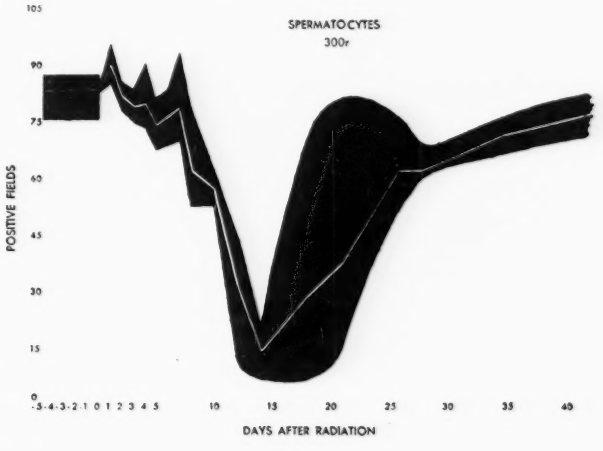

CHART 2.—Numerical frequency of spermatocytes plotted against time following direct radiation of testis, 300 r.

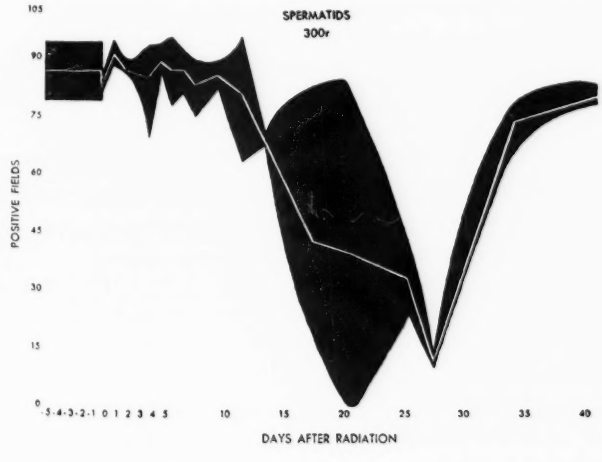

CHART 3.—Numerical frequency of spermatids plotted against time following direct radiation of testis, 300 r.

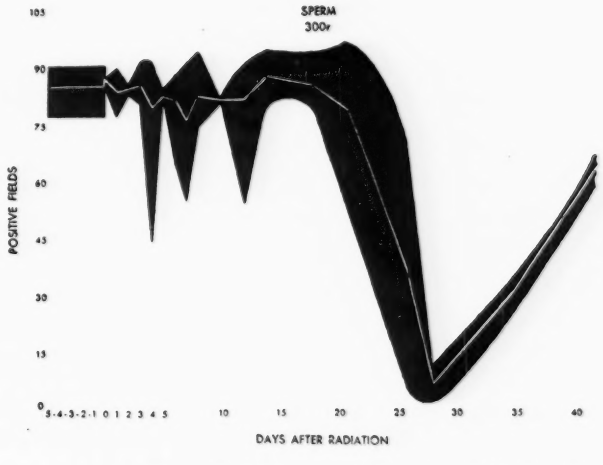

CHART 4.—Numerical frequency of sperm plotted against time following direct radiation of testis, 300 r.

sperm had not returned to normal, but there was a normal frequency of spermatogonia.

Chart 1 graphically illustrates that there is a period between the 10th and 27th day of marked numerical variation in the frequency of appearance of spermatogonia in the testes following 300 r. The white line represents the average number of times in which these epithelial elements appeared in 100 fields at any given interval after radiation. The black area represents the extent of numerical variation. Attention should be called to the similarity of the descending slope of the curves of each of the germinal elements. The period of greatest biological variation is noted in Charts 2, 3, and 4 for spermatocytes, spermatids, and sperm. It is in the period of 10–28 days.

A study of mice subjected to over-all radiation at 300 r was made for purposes of contrast with the results obtained from direct radiation. With the use of the same computative method, there was little variation between these two groups in the average frequency of the germinal elements at stated intervals at this dosage.

Our second set of controls, namely, those animals which were subjected only to the same manipulative treatment as the radiated animals, but which were not radiated, shows no significant difference in total count or extent of numerical variation as compared to those not subjected to immobilization.

For the entire period of observation the Sertoli cells, the interstitial cells, and the connective tissue did not reveal any marked pathological change.

Illustrations are submitted of median cross sections of tubules, showing in Figure 1 the condition observed most frequently up to 1 day with all the germinal elements; in Figure 2, the condition at 5–7 days, showing a general loss of spermatogonia; in Figure 3, the condition at 14 days, showing a general loss of spermatogonia and spermatocytes; in Figure 4, the condition at 26–28 days, showing a high incidence of the presence of sperm, general absence of spermatocytes and spermatids, and the occasional appearance of spermatogonia; and in Figure 5, the condition at 28 days, when spermatids and sperm are at the lowest incidence—but spermatogonia now have almost a normal frequency, and spermatocytes are occasionally seen.

DISCUSSION

The purpose of this investigation was to study the effects of direct x-radiation on the testis, with special concern for fundamental problems of growth, regeneration, and differential response of cellular elements to radiation, in the hope that this would contribute to the knowledge of neoplasia. The effects of localized and over-all radiation given under similar conditions have been used for purposes of comparison. Observations are being made on results from both higher and lower doses, with other conditions the same. The results are to be submitted later.

This system of noting the germinal elements at intervals after radiation does not indicate whether any of the spermatocytes are capable of meiosis or whether spermatids are able to metamorphose into viable sperm in the 21-day period post-radiation; nor is it known how long sperm will remain viable. The production of normal young by a normal female mated with a radiated male is the test for determining if a radiated male carries unaffected germ cells. Such successful matings were obtained with radiated males and normal females, but the data appear insufficient for conclusions, and it is planned to study this matter further.

The general belief that spermatogonia are the most sensitive to radiation is partially confirmed in our results by the fact that they begin to disappear shortly after radiation, while the spermatocytes, spermatids, and sperm follow at progressively later periods. This is consistent with the observation of other workers.

As shown in Chart 1, the incidence of tubules revealing spermatogonia begins to increase in the eighth day. Charts 2–4 show that the spermatocytes, spermatids, and sperm begin to increase at progressively later periods. A comparison of these figures shows that the spermatogonia are on the increase, while the other types of germinal cells are on the decrease. This allows an estimate of the time needed under the conditions of the experiment for a spermatogonium to have developed into a sperm—namely, the time elapsed from 5–7 days to 28 days or, approximately, 22 days.

As has been stated earlier, the determination of the sensitivity of these germ cells to radiation has been based on the time of their decline in occurrence. In other words, the spermatogonia are said to have greater radio-sensitivity because they are the first to become reduced in frequency. The results of this work indicate that it takes longer for the spermatocytes, spermatids, and sperm to show response, but when the reaction takes place the rate of disappearance is approximately the same. The meiotic stages of the spermatocytes and the metamorphosis of the spermatids and sperm represent stages of differentiation which are supposed to be relatively radio-resistant. In our case, however, the differentiation is marked only by a

greater time interval required for the onset of the reaction; but, when it begins, it proceeds at the same rate as that for the spermatogonia.

A dose of 300 r does not cause the complete disappearance of the spermatogonia in the 5–7 day period. This suggests the possibility that we are not dealing with a homogeneous population, as there are always some spermatogonia unaffected by radiation.

It was noted that the counts made during the early intervals showed irregularities, as indicated by the sharp peaks and depressions. These, however, become more infrequent and less marked with time. Reference to the figures will show they are much less marked after 21 days. This suggests that in the early intervals the environment in which these cells exist is unfavorable for mitotic activity. By 21 days, however, conditions have modified toward normal. The situation may be analogous to a colchicine-like effect, where mitoses are arrested at metaphase and cannot proceed further normally while the drug is present.

In the first few days, if a cell initiates division, it rapidly undergoes necrotic liquefaction and disappears. In other words, it is suggestive that the reservoir of gonia present at the beginning is partially depleted as repeated mitosis is attempted. These cells were not all in prophase when irradiated, yet they showed the liquefaction effects only on attempted division. A somewhat similar situation was reported by Glücksmann and Spear (6), who showed that after irradiation of tissue there is seen a succession of degenerate cells, which represent cells attempting to enter division and breaking down in prophase. The lysis of a cell in division would be more complete and would leave fewer traces than would a more viscous resting cell. This would conform with the observations of Liebow, Warren, and DeCoursey (10) and others that radiated cells in the testis have a tendency to disappear completely. R. E. Zirkle (15) reviewed the literature and emphasized the need of further study of chemical changes following radiation.

The presented data from studies of over-all radiation at this low dosage confirm and complement our findings for localized radiation.

The suggestion that degeneration of Sertoli cells was a factor in the loss of spermatids and sperm was not demonstrated by our method at the dose used. A random quantitative sampling of the number of Sertoli cells in 15 cross sections of tubules indicated from 24–32 cells in each of the five normal animals. An equivalent count made on the same number of radiated animals at intervals of 2, 5, 12, 21, 28, and 42 days gave the same result. These radiated Sertoli cells did not show shrinkage, separation from the membrane, or other evidence of pathology. This lack of pathology was also apparent in the interstitial cells and in other connective tissue such as the blood vessels or the capsule of the tubules.

SUMMARY

Testes of pure line C57 black adult mice, 18–25 gm., were subjected to a single dose of x-radiation at 300 r, both direct and total-body.

The evaluation of radiation effects has been based on a quantitative random sampling of the germinal elements of the testis, quantitated with respect to frequency of cell population types plotted against time after radiation.

Evidence is presented to show that a small percentage of all germinal elements persist after a dose of 300 r. The period of least frequency of spermatocytes is around 14 days; that of spermatids and sperm, 28 days. The data indicate that it takes 21–22 days for a gonium to develop into sperm.

The slopes of the curves in terms of time and progressive loss of each of the germinal elements are roughly the same. This suggests that each of the germinal elements is affected to the same degree but at different intervals.

The period of greatest biological variation is from 10 to 28 days for each of the four germinal elements.

A single dose of 300 r does not cause the disappearance of Sertoli cells, hyperplasia of the interstitial cells, nor is connective tissue visibly affected in the 12-week interval included in this study.

REFERENCES

1. BLOOM, W. Histopathology of Irradiation from External and Internal Sources, pp. 55, 568–69. 1st ed. New York, Toronto, and London: McGraw-Hill Book Co., 1948.
2. CHALKLEY, H. W. Method for the Quantitative Morphologic Analysis of Tissues. J. Nat. Cancer Inst., **4**:47–53, 1943.
3. ESCHENBRENNER, A., and MILLER, E. Quantitative histologic Analysis of the Effect of X-Radiation on the Interstitial Tissue of the Testes of LAF_1 Mice. J. Nat. Cancer Inst., **6**:343–48, 1946.
4. FOGG, L., and WARREN, S. Some Cytologic Effects of Repeated Doses of Radiation on Mouse Sarcoma 180. Cancer Research, **2**:517–20, 1942.
5. HOWARD, R. P.; SNIFFEN, R. C.; SIMMONS, F. A.; and ALBRIGHT, F. Testicular Deficiency: A Clinical and Pathologic Study. J. Clin. Endocrinol., **10**:121–86, 1950.
6. GLÜCKSMANN, A., and SPEAR, F. G. The Effect of Gamma Radiation on Cells *in Vivo*. Part 2. Brit. J. Radiol., **12**: 486–98, 1939.
7. JOEL, C. A. Veränderungen der Hypophyse nach Röntgenbestrahlung der Hoden. Schweiz. med. Wchnschr., **23**: 795–96, 1942.

8. Lea, E. Actions of Radiation on Living Cells, pp. 364–84. New York: The Macmillan Co.; Cambridge: At the University Press, 1947.

9. LeRoy, G. V. Medical Sequelae of Atomic Bomb Explosion. J.A.M.A., **134**:1143–48, 1947.

10. Liebow, A.; Warren, S.; and DeCoursey, E. Pathology of Atomic Bomb Casualties. Am. J. Path., **25**:919, 924, 928, 1949.

11. Mathis, A.; Brooks, R.; and Schneiderman, H. The Effect of X-Irradiation on Hyaluronic Acid. Atomic Energy Project 91121-4-2-10004, 1949.

12. Tullis, J., and Barrow, J. The Sequence of Cellular Response to Injury in Mice Exposed to 1100 r Total Body X-Radiation. Naval Medical Research Inst., **23**:NM 007 039, 1949.

13. Van Wagener, G. Histological Changes in the Male Rat Hypophysis Following Degeneration of the Germinal Epithelium. Anat. Rec., **29**:398–99, 1924–25.

14. Warren, S. Effects of Radiation on Normal Tissues. Arch. Path., **35**:121–39, 1943.

15. Zirkle, R. E. Relationships between Chemical and Biological Effects of Ionizing Radiations. Radiology, **52**: 846–55, 1949.

Fig. 1.—Median cross section mouse testis tubule as seen 3–4 hours post-radiation, 300 r. All the germinal elements are present. Mag. ×444.

Fig. 2.—Median cross section mouse testis tubule as seen 5 days post-radiation, 300 r. The spermatogonia do not appear. Mag. ×444.

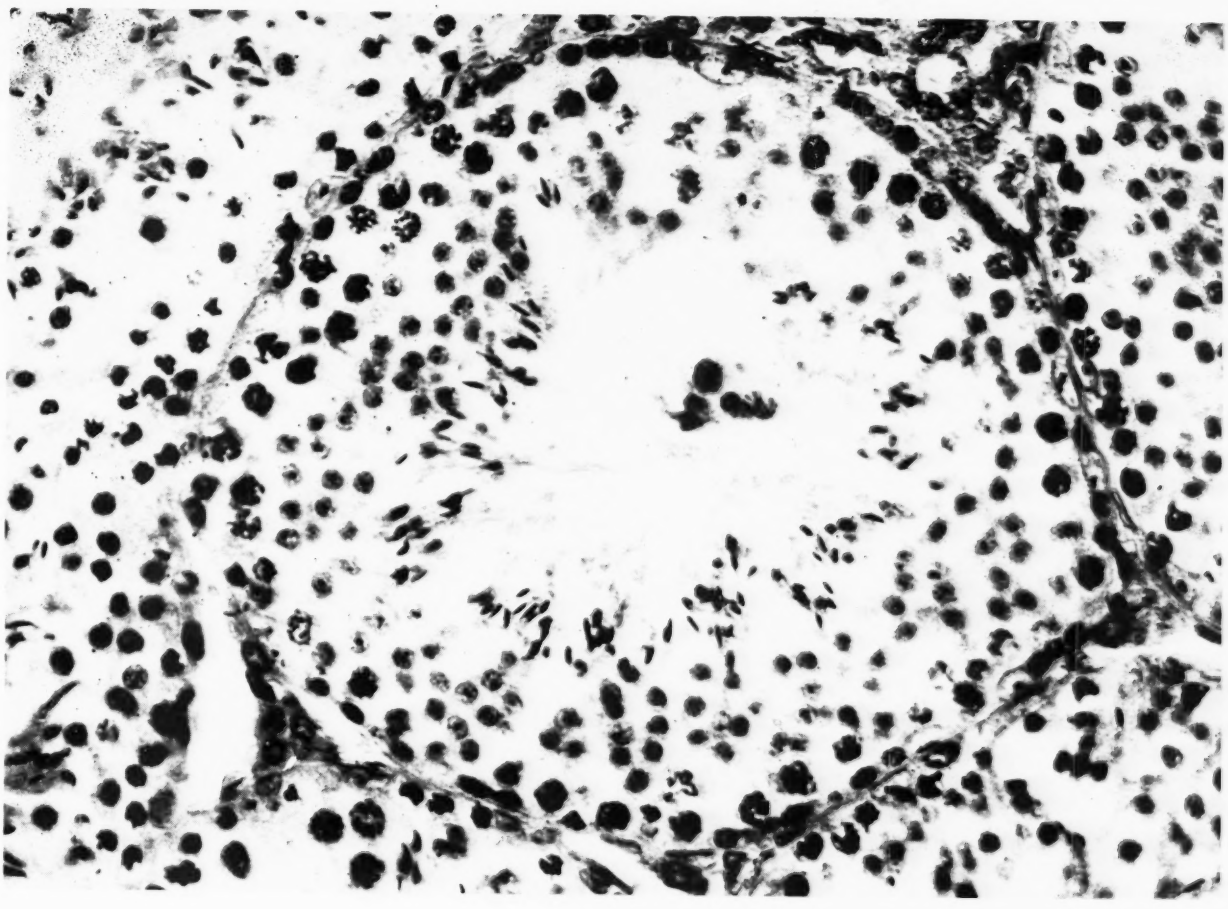

FIG. 1

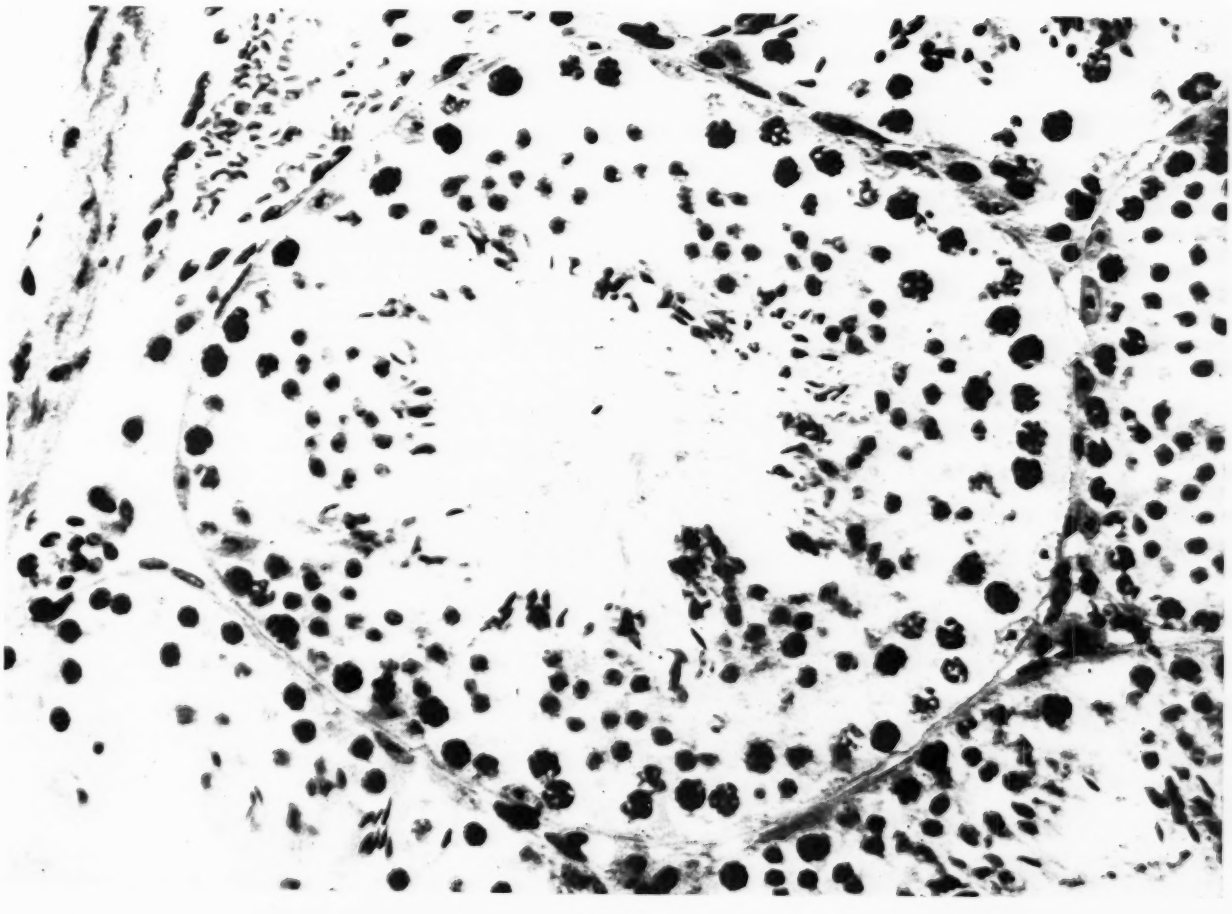

FIG. 2

FIG. 3.—Median cross section mouse testis tubule as seen 14 days post-radiation, 300 r. The spermatocytes are absent; spermatids and sperm are still present. Mag. ×444.

FIG. 4.—Median cross section mouse testis tubule as seen 21 days post-radiation, 300 r. At this period sperm are still present. In some tubules, typified by this illustration, spermatogonia can now be identified. Mag. ×444.

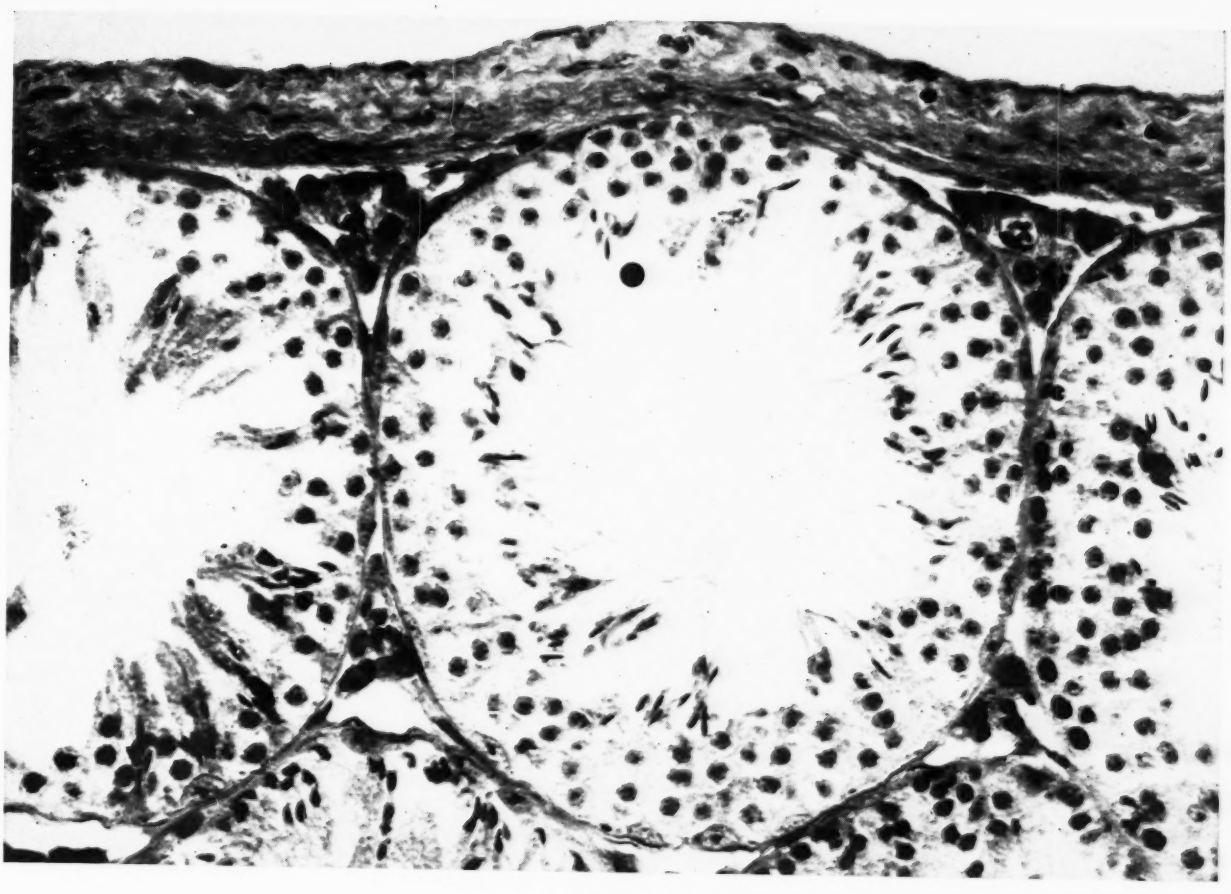

FIG. 3

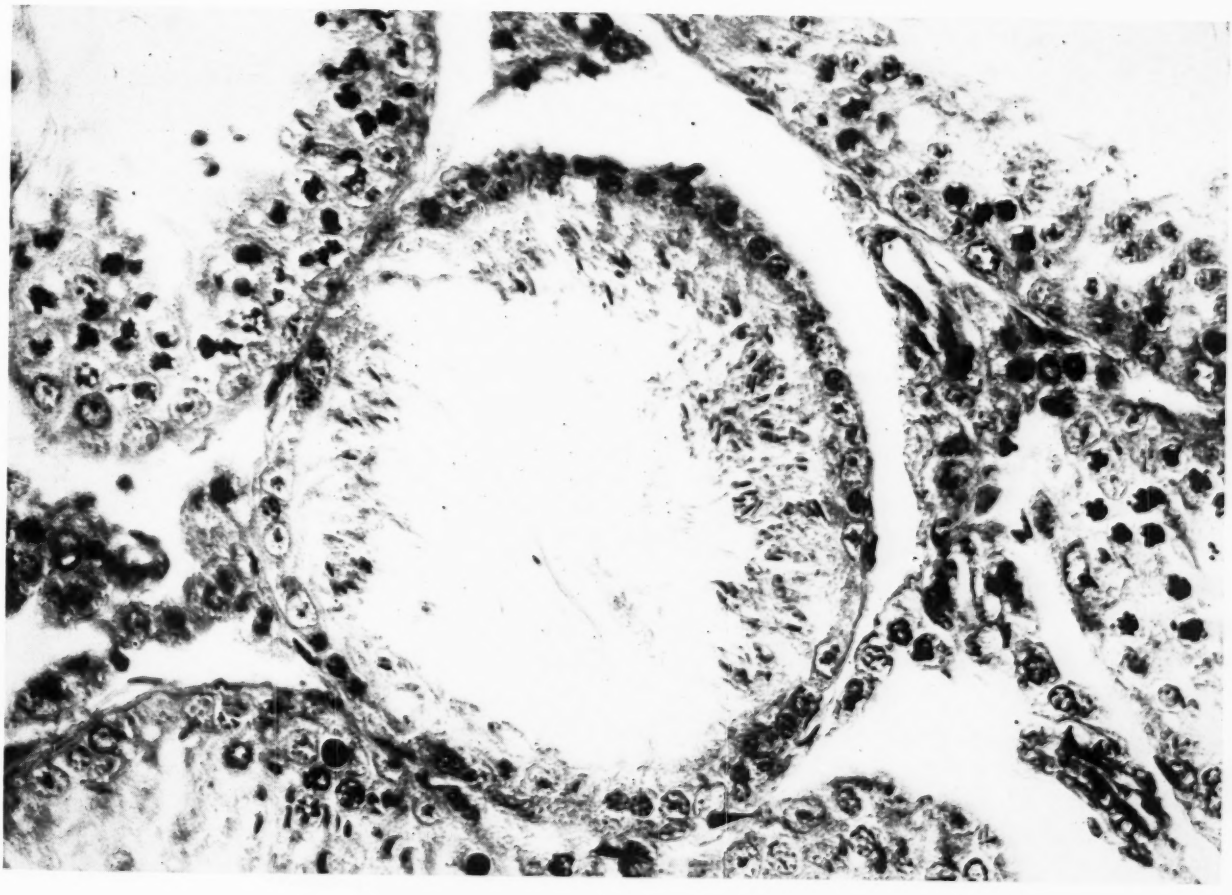

FIG. 4

FIG. 5.—Median cross section mouse testis tubule as seen 28 days post-radiation, 300 r. Spermatids and sperm are absent. Spermatogonia and spermatocytes now are present. Mag. ×444.

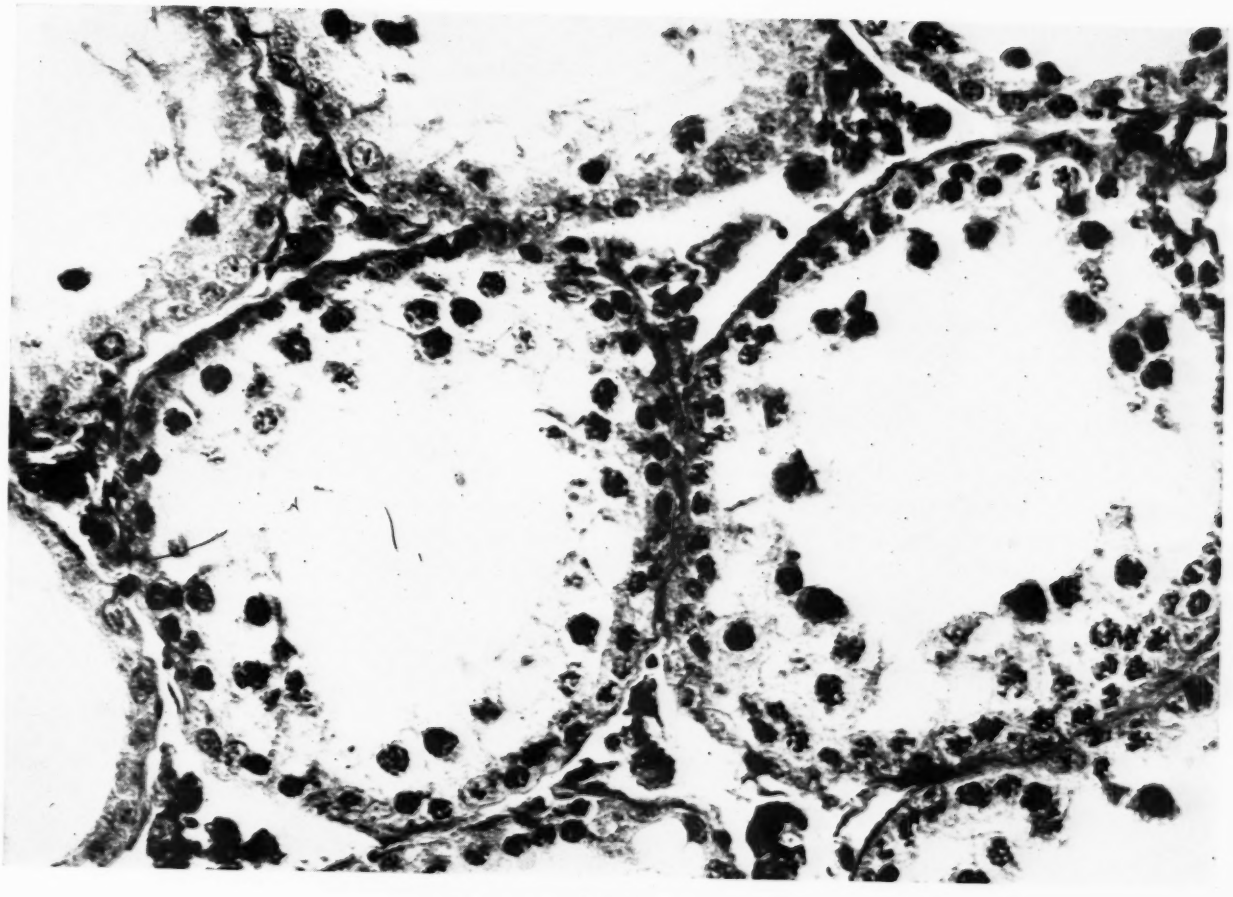

FIG. 5

The Development of Lymphatic and Myelogenous Leukemia in Wistar Rats Following Gastric Instillation of Methylcholanthrene

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A number of cases of lymphatic and myelogenous leukemia have appeared in the course of various experiments we have been conducting with Wistar rats. The animals affected were involved in several different types of investigations and had been subjected to procedures in which the only common denominator was the intragastric instillation of 20-methylcholanthrene.[1]

In the published reports of experiments with Wistar rats, only one case of induced leukemia has previously been recorded. This was noted by Murphy and Sturm (12) in 1941, when they observed the development of lymphatic leukemia in an animal of this strain following the accidental injection of dibenzanthracene into a lymph node. There are no cases on record of the development of spontaneous lymphatic or myelogenous leukemia in a rat of this stock.

Before 1936, leukemia had not been reported in any strain of rat. In that year, Wilens and Sproul (23) described eleven instances of myelogenous and one of lymphatic leukemia in an inbred strain of Osborne-Mendel albino rats. Two years later Rask-Nielsen (14) found, among 30 old white rats of uncertain origin, one animal with a large abdominal tumor composed of "pathological myeloblasts." Blood smears from this animal were normal except for 6 per cent of the white cells which she regarded as "immature pathological myeloblasts." Rask-Nielsen (14) was able to transfer the tumor to other rats, but at no time did "pathological myeloblasts" appear in the peripheral blood of these animals. The following year, Oberling, Guérin, and Guérin (13) reported six cases of lymphatic and three of myelogenous leukemia among 6,000 rats. These were observed only in older animals, the youngest 17–18 months of age, and the majority in animals more than 28 months old. In 1940, Arai (1) found, in a group of 500 rats of different strains, 27 animals with spontaneous tumors and noted one instance of chronic myelogenous leukemia.

Ratcliffe (15) in that same year described 273 tumors in a large colony of Wistar rats. Among these, there were two lymphoblastomas of the mediastinum, one lymphoid tumor of the thymus, and two lymphosarcomas, but none produced a leukemia. More recently, Farris and Yeakel (6) reported nine instances of reticulum-cell sarcoma in 1,000 autopsies on Wistar rats, without mention of a single case of leukemia. Similarly, Bullock and Curtis (3) found no leukemia in 78 animals with sarcoma arising in the mesenteric lymph nodes, among 489 spontaneous tumors found in 2,450 rats whose original source was apparently unknown. Although described as spontaneous tumors, it is difficult to accept them as such without reservation, since all the animals except 23 had been fed ova of *Taenia crassicollis*, the tapeworm of the cat, a procedure which these authors had described previously (4) as a simple method for producing sarcoma of the liver in rats.

Other efforts to produce leukemia in rats have been made. Bernard (2), using the same approach that Thomsen and Engelbreth-Holm (22) employed in fowls, was unsuccessful in his attempt to produce leukemia in rats by injecting carcinogenic tar into the bone-marrow. Storti and Storti (21) also failed after injecting 3,4-benzpyrene into the femoral bone marrow of 100 rats. Gennaro and Grazia (8) observed the development of one case of lymphatic and one of myelogenous leukemia among fifteen rats whose skin they painted with a 1 per cent solution of benzpyrene in benzene. In 1940, Ito (9) found myelogenous leukemia in one of twenty white rats, not identified as to strain, following the feeding of *o*-aminoazotoluene and methylene blue.

In our experiments, eight cases of leukemia appeared in a total of 59 Wistar rats under study, and a successful transfer by intraperitoneal injection of spleen emulsion was effected to two of three additional young rats from our colony.

[1] Eastman Kodak Co.

Received for publication July 24, 1950.

MATERIALS AND METHODS

All experiments were performed on Wistar strain albino rats grown in our own colony in which inbreeding has been carefully avoided. The cases reported here were contributed by six groups of animals, consisting of intact and castrated males and females.

Two of these groups were maintained on a calcium-free diet, two on an approximation of the "Roffo diet," and two on our colony Rockland diet. The only procedure common to all six groups was the administration of methylcholanthrene by stomach catheter (18).

EXPERIMENTS AND RESULTS

CASE 1, LYMPHATIC LEUKEMIA.—

Experimental method.—Calcium-free diet plus methylcholanthrene in castrated male.

A male rat was castrated at the age of 7 weeks and placed on the calcium-free diet recommended by Zucker (24) for the production of gastric ulceration of the antral portion of the rat's stomach. This diet was replaced for short periods with our colony Rockland diet, because young rats could not be maintained indefinitely on the calcium-free diet (17). Once the experimental diet was started, the animal received 2 mg. of methylcholanthrene dissolved in 0.5 cc. of olive oil, administered by stomach catheter (17), daily for 6 days of the week.

The animal was found dead in its cage at the age of approximately 12.5 months, after having received methylcholanthrene as described for nearly 11 months.

A blood count taken 15 days before death showed the following: R.B.C., 5,330,000; W.B.C. 38,300; differential: polymorphonuclears 8 per cent, lymphocytes 52 per cent, lymphoblasts 40 per cent, 1 nucleated R.B.C./100 W.B.C.

Bone-marrow smear showed the bone marrow to be largely replaced by lymphocytes.

Autopsy.—The significant gross findings included enlarged palpable lymph nodes in both axillary and inguinal regions, which had been noted on clinical examination 5 days before death. The abdominal lymph nodes were also enlarged, as were the nodes in the region of the thymus. The liver appeared larger than normal. The spleen was enlarged and measured 5 cm. × 1.4 cm.

Histology.—Sections of the liver showed an infiltration of rather pleomorphic hyperchromic cells, some of which resembled lymphocytes. The infiltration was diffuse throughout the sinusoids and was also present in the form of small nodules which did not have a definite architectural relationship, although most portal triads were infiltrated. The normal architecture of the spleen was destroyed by an infiltration of cells which were similar to those described in the liver. There was also a moderate degree of hemosiderosis. Sections from several lymph nodes showed complete destruction of the normal structure, with replacement of the normal cells by cells similar to those described above. The infiltration was not limited by the capsule, and the same type of cells was also present in the perinodal fat and connective tissue. A tumor mass adjacent to the undersurface of the liver proved to be the pancreas, markedly infiltrated by large numbers of cells not as pleomorphic as those found in the liver. This infiltration almost completely replaced the pancreatic tissue. A kidney section showed an extremely heavy infiltration of cells similar to those infiltrating the pancreas. The tumor cells almost completely replaced the interstitial tissue in the cortex, and the number of nephrons appeared to be reduced. The tumor cells also infiltrated the pelvic fat. The brain was infiltrated by cells similar to those already described, which were grouped in the subarachnoid space and in the adjacent brain substance. This infiltration was not pronounced. Peroxidase stain was negative in all the tissue sections in all the cases of lymphatic leukemia.

This animal died at the age of 12.5 months of lymphatic leukemia which was first detected by blood count after the animal had received methylcholanthrene for approximately 10.5 months. Since no previous blood counts had been done, it is not possible to conjecture how much earlier the leukemic state had developed.

Seven additional rats with lymphatic leukemia are included in this report. While the essential experimental details are given below, the blood counts done in the course of the study are tabulated in Table 1. Since the various tissues in these animals were infiltrated by cells that closely resembled those described in the infiltrations in Case 1 and varied only in the degree of organ involvement, the gross and histologic features have been listed in Tables 2 and 3.

CASE 2, LYMPHATIC LEUKEMIA.—

Experimental method.—Colony Rockland diet plus methylcholanthrene in spayed female.

This animal was spayed at the age of 6 weeks and placed on the methylcholanthrene schedule described in Case 1. It died, at 8 months of age, of lymphatic leukemia, after it had received methylcholanthrene for 6.5 months. Since a blood count, taken only 16 days before death, failed to show any evidence of leukemia, this result suggests that the transformation of the normal cell to the malignant cell occurred rather suddenly, as pointed out by McEndy, Boon, and Furth (11) in their studies

with methylcholanthrene-induced leukemia in mice.

We have presented elsewhere (19) suggestive evidence that methylcholanthrene administered by stomach catheter to the lactating mother may be transferred to the offspring. We are also investigating whether the administration of the carcinogen during pregnancy will exert any influence on the offspring. In one experiment of this type, normal male and female rats were mated, and impregnation of the female was assumed to have occurred upon the finding of the vaginal mucus plug. Gastric instillation of methylcholanthrene as described for Case 1 was started when such a plug was found and continued until the day of delivery, or until the time that we could be certain pregnancy had not occurred. All animals in this group were maintained on our colony Rockland diet. The following two cases were contributed by this group.

CASE 3, LYMPHATIC LEUKEMIA.—

Experimental method.—Colony Rockland diet plus methylcholanthrene in intact female.

This female, at 4 months of age, was placed on the methylcholanthrene schedule for a period of 28 days. She had not become pregnant. Eighty-two days after the gastric instillation of methylcholanthrene was discontinued, the animal was found dead in its cage. No blood counts had as yet been taken.

CASE 4, LYMPHATIC LEUKEMIA.—

Experimental method.—Colony Rockland diet plus methylcholanthrene in intact female.

This rat, at 5 months of age, received methyl-

TABLE 1

BLOOD COUNTS OF LEUKEMIC RATS

Case	Days before death	Hemoglobin (per cent)	White blood count	Polymorphs (per cent)	Lymphocytes (per cent)	Lymphoblasts (per cent)	Monocytes (per cent)	Eosinophils (per cent)	Basophils (per cent)	Nucleated reds (per 100 W.B.C.)	Myeloblasts (per cent)
1	15		38,300	8	52	40				1	
2	16	13.5	17,000	29	53		13		4		1
3	Not done										
4	30	13.5	15,000								
5	65	12	8,700	28	71				1		
6	37	13.5	13,800			1					
	1	10.5	150,000	1	5	94					
6A	13	13.5	17,000	33	57	2	8				
	0	10.8	340,000	1	4	95					
6B	25	15	17,700	19	67	1	8				
	0	8.7	47,000		8	92					

TABLE 2

TISSUE CHANGES FOUND AT AUTOPSY*

Case	Lymph nodes: Axillary	Inguinal	Abdominal	Thoracic	Liver	Spleen
1	++	++	++	++	++	5×1.4 cm.
2					+++	5.2×1.5 cm.
3			++			6×1.5 cm.
4			++		+++	7×2 cm.×1 cm.
5	++		++	++	pale+fatty	5.7×1.4 cm.
6			++		++	5×1.5 cm.
6A	++	+	++	++		4.5×1.2×0.6 cm.
6B			++			3.0×1.3×1.8 cm.

* Degree of enlargement indicated by + signs.

TABLE 3

TISSUES INFILTRATED WITH LEUKEMIC CELLS*

Experiment	Brain	Heart	Lung	Liver	Stomach	Small intestine	Pancreas	Kidney	Uterus	Spleen	Lymph node	Thymus	Adrenal gland	Salivary gland	Bone marrow	Thyroid
1	+			++			++++	+++		++++	++++				N.e.	
2			+++	++		+++				++++	++++	N.e.†	+		+++	
3			++	+++				+		++++	++++	N.e.	+		N.e.	
4				++++				+		++++	++++	N.e.			N.e.	
5			+	+++				+		++++	++++	N.e.			N.e.	
6	+	+	+	++	++	++	++	+		++++	++++	N.e.			+++	++
6A		+	+	+	+					+++	++++			+	+++	
6B			+	+	+					++++	++++	++++			+++	

* Degree of infiltration + to ++++.

† N.e. = not examined.

cholanthrene for 21 days, at which time it gave birth to its litter. Six days later she killed all the newborn.

A blood count taken approximately 5.5 months after the last dose of carcinogen is shown in Table 1.

The animal was found dead in its cage just 1 month later, 200 days after completing its course of methylcholanthrene. A blood smear taken from the heart blood showed a large number of lymphoblasts. It was 1 year of age at the time of death.

Roffo (16) has reported the development of adenocarcinoma in the rat's stomach following a diet of bread and milk to which was added an equal amount of either lard, beef tallow, mutton tallow or of olive oil which had been heated to 350° C. for ½ hour. In the course of our studies, we attempted to repeat Roffo's conditions in two groups of rats.

The diet, No. 74, was constructed in an effort to simulate as closely as possible that used by Roffo, exact details of which could not be gleaned even though his published reports are extensive. Diet 74 had the following composition: 1,948 gm. of milk, 340 gm. of Kellogg's All Bran, and 400 gm. of white bread.

With this diet, one group of animals received methylcholanthrene in 1 cc. of unheated olive oil, on a schedule similar to that in Case 1, while the other was given methylcholanthrene in 1 cc. of heated olive oil. Each group contributed one case of lymphatic leukemia as described below.

Case 5, Lymphatic Leukemia.—

Experimental method.—Diet 74 plus methylcholanthrene in intact male.

This male, when 8 weeks old, was placed on experimental diet 74 with 2 mg. methylcholanthrene in 1 cc. of unheated olive oil, administered by stomach catheter, on a schedule similar to that used in Case 1.

A blood count taken 6 months after the carcinogen and diet were started is shown in Table 1. Two months later the 10-month-old animal was found dead in its cage, after having received methylcholanthrene for the last 7.5 months.

Case 6, Lymphatic Leukemia.—

Experimental method.—Diet 74 plus methylcholanthrene in heated olive oil in intact male.

This male rat was placed on diet 74 at 8 weeks of age, and 5 days later began to receive methylcholanthrene dissolved in olive oil that had been heated to 350° C. for ½ hour. A blood count (Table 1) taken 1 day before death is in sharp contrast with one taken 5.5 weeks earlier. It was sacrificed at the age of 14.5 months after having received methylcholanthrene in heated olive oil for 12.5 months.

At autopsy, after a section of spleen was taken for histologic examination, the remainder of the organ was ground with a mortar and pestle with 5 cc. of normal saline. A 1-cc. portion of suspended cells was injected intraperitoneally into each of three normal rats, 6 weeks of age, from our stock colony. Two of these transfers (Cases 6A and 6B) were successful.

Case 6A, 19 days after injection, showed enlarged inguinal, maxillary, and sublingual lymph nodes. Blood counts 6 and 19 days after the injection of the splenic cells are shown in Table 1.

The animal was sacrificed after the second count was taken.

Case 6B was sacrificed 60 days after the intraperitoneal injection of the leukemic spleen cells. The pertinent data are shown in the charts.

Case 7, Myelogenous Leukemia.—

Experimental method.—Calcium-free diet plus methylcholanthrene in castrated male.

A male rat was castrated at 7 weeks of age and placed on the same diet and methylcholanthrene schedule as the animal in Case 1. It was sacrificed at 16 months of age when the peripheral blood count showed a picture compatible with that of myelogenous leukemia.

Nine months after the carcinogen was started, a small mass was palpated in the right anterior loin fold, corresponding to what we have described in a previous publication as a type B tumor (17). Three months later, a similar mass was palpated in the left anterior loin fold.

A blood count at that time was still within normal limits: R.B.C., 8,830,000; W.B.C., 10,000; differential: polymorphonuclears 27 per cent, lymphocytes 73 per cent (occasional large lymphocytes).

Six weeks later additional similar masses were palpated in each posterior axillary fold.

A blood count 2.5 months after the first count showed R.B.C., 2,590,000; W.B.C., 70,800; differential: polymorphonuclears 35 per cent, stab forms 9 per cent, myelocytes 32 per cent (15 per cent ring forms), myeloblasts 14 per cent, lymphocytes 10 per cent, 3 nucleated R.B.C./100 W.B.C.

There was an increase of granulocytes in the bone marrow based on peroxidase stain. A peroxidase stain of all tissues was positive.

Autopsy.—The significant gross findings were an enlarged liver and spleen, the latter measuring 6 × 1.8 cm. Two enlarged abdominal lymph nodes were also noted.

Histology.—Sections of liver showed an appreciable infiltration of cells of different shapes. The nuclei varied from round forms, through horseshoe shapes, to the typical lobulated nuclei of the ma-

ture polymorphonuclear leukocytes. The infiltrating cells were particularly concentrated around the portal triads, although a moderate number of similar cells could be seen in the sinusoids. The architecture of the liver was relatively undistorted. The sinusoids were dilated, but the triads were readily identified. The parenchymal cells showed granularity of the cytoplasm with some vacuolization. A moderate number of binucleated cells were observed. The architecture of the spleen was distorted, and follicles could not be recognized. The parenchyma was replaced by large numbers of cells identical in appearance to those infiltrating the liver. A moderate number of giant cells which were doubtlessly megakaryocytes were scattered throughout the section with no well defined architectural distribution. The areas around the larger bronchi in the lungs were moderately infiltrated by cells similar to those described above. In many areas there was considerable thickening and broadening of the alveolar septa. This broadening was apparently due to infiltration of the septa by cells similar to those described. The renal architecture was undistorted, and the glomeruli were normal in number and appearance. Varying degrees of parenchymatous degeneration were apparent, however, and the cells were swollen and bulged into the lumina, many of which were obliterated. The cortex was lightly infiltrated by cells similar to those already described, with the greatest concentration noted around the larger arterioles. The perirenal fat was rather heavily infiltrated. The adrenal gland was infiltrated by cells beneath the capsule at one pole. There was a light scattering of similar cells in the sinusoids of the cortex. The medulla was uninvolved. Sections of lymph nodes from the vicinity of the appendix and right loin, the mesenteric nodes, the maxillary lymph nodes and peripancreatic nodes all showed destruction of the normal follicular architecture, although the sinusoids were still recognizable. The nodes consisted principally of cells similar to those infiltrating the organs previously described. The pancreas was markedly infiltrated by cells which seemed to originate in the adjacent peripancreatic nodes. Except for this cellular infiltration, the pancreatic tissue was normal. There was no infiltration of the maxillary gland. A section of thyroid, including the trachea and surrounding muscle, had cellular infiltrations in the fat and connective tissue immediately adjacent to the glandular tissue. Peroxidase stain was positive on all the infiltrated areas of the tissues described.

This animal was 14.5 months of age and had received methylcholanthrene for slightly longer than 13 months when the leukemic state was evidenced in the blood count. Since a normal blood picture had been obtained approximately 2.5 months earlier, the development of myelogenous leukemia in this rat is placed between 10.5 and 13 months after the beginning of methylcholanthrene administration.

CASE 8, MYELOGENOUS LEUKEMIA.—

Experimental method.—Calcium-free diet plus methylcholanthrene in intact male.

This male, at 6.5 weeks of age, was placed on the calcium-free diet and methylcholanthrene schedule identical with that used in Case 1 and was sacrificed 14 months later.

A blood count was taken approximately 10.5 months after the methylcholanthrene was started and showed: R.B.C., 6,030,000; W.B.C., 14,600; differential: polymorphonuclears, 47 per cent; eosinophils, 2 per cent; lymphocytes, 51 per cent.

Three and one-half months later, a blood count gave the following results: hemoglobin, 9.7 gm.; W.B.C., 109,800; differential: polymorphonuclears 36 per cent, stab forms 16 per cent, myelocytes 37 per cent (25 per cent ring forms), myeloblasts 3 per cent, lymphocytes 8 per cent, 2 nucleated R.B.C./100 W.B.C.

It was sacrificed the next day.

Autopsy.—The salient gross findings were an enlarged spleen, measuring 6.4 × 1.6 cm. and weighing 4.5 gm., and an enlarged liver which weighed 9.9 gm. There were no obviously enlarged lymph nodes, and the kidneys grossly appeared to be normal.

Histology.—Sections of the liver, spleen, and lungs showed an infiltration by cells similar to those described in Case 7. The kidneys, adrenal gland, and testicles were not infiltrated. A peroxidase stain of all the tissues was positive.

DISCUSSION

In the material that is the basis of this report, we have seen the development of two examples of myelogenous leukemia and six of lymphatic leukemia in a total of 59 Wistar rats under study.

These cases were contributed from six groups of animals on various diets in which the administration of methylcholanthrene by stomach catheter was the only procedure common to all. The length of time for which the carcinogen was administered before the leukemic state was detected ranged from approximately 1 to 14 months. In four of the eight rats, the leukemic state was suspected first from the blood count. In another animal, a blood count approximately 2 months before it died, and, in still another, a blood count 16 days before death, failed to show any diagnostic changes.

Peripheral lymph nodes were palpable clinically

in four of our animals with lymphatic leukemia.

From one case of lymphatic leukemia which developed in our series, a successful transfer to two of three young Wistar rats from our stock colony was effected by the intraperitoneal injection of splenic tissue brayed in normal saline. Extensive transfer studies have been carried out subsequently and will be reported separately.

Methylcholanthrene applied percutaneously has induced and hastened the development of leukemia in mice. Thus, Kirschbaum, Strong, and Gardner (10) found that leukemia appeared earlier in a strain of mice in which spontaneous leukemia was common when methylcholanthrene was applied percutaneously. The same procedure had little effect, however, in mice with no predisposition. On the contrary, Furth and Barnes (7) were able to show that even mice of a low leukemic stock could readily be rendered leukemic by the skin application of the same carcinogen. Furthermore, McEndy, Boon, and Furth (11) induced 72 cases of leukemia in mice by this method, of which 33 were lymphoid leukemia and only 2 myeloid. Of particular interest is the failure of Stewart and Lorenz (20) to find any instance of leukemia in their extensive feeding experiments with methylcholanthrene in mice.

SUMMARY

Six cases of lymphatic and two cases of myelogenous leukemia developed in a group of 59 Wistar rats under a variety of experimental conditions but in which all animals received methylcholanthrene by stomach catheter. The carcinogen was administered for periods of 1–14 months. From one case of lymphatic leukemia, the disease was transferred to two of three young colony rats by intraperitoneal injection of splenic cells suspended in normal saline.

REFERENCES

1. Arai, M. Über die spontanen Geschwülste bei weissen Ratten. Gann, **34**:137–43, 1940.
2. Bernard, J. Effets des injections intra-spléniques et intraganglionnaires de goudron chez le rat. Sang, **9**:866–72, 1935.
3. Bullock, F. D., and Curtis, M. R. Spontaneous Tumors of the Rat. J. Cancer Research, **14**:1–115, 1930.
4. ———. The Experimental Production of Sarcoma of the Liver of Rats. Proc. N.Y. Path. Soc., **20**:149–75, 1920.
5. Farris, E. J., and Griffith, J. Q. (ed.). The Rat in Laboratory Investigation. 2d ed. Philadelphia: J. B. Lippincott Co., 1949.
6. Farris, E. J., and Yeakel, E. H. Spontaneous and Transplanted Reticulum Cell Sarcomas in Wistar Rats. Am. J. Path., **20**:773–81, 1944.
7. Furth, J., and Barnes, W. A. Differences between Malignant Blood Cells from Induced and Spontaneous Leukemias of Mice. Cancer Research, **1**:17–22, 1941.
8. Gennaro, A. De, and Grazia, A. Di. Sull' insorgenza di leucemie in topi trattati con idrocarburi policiclici on cogeni. Haematologica, **18**:707–26, 1937.
9. Ito, S. Über die Entstehung der Leukaemie bei Ratten. Gann, **34**:133–37, 1940.
10. Kirschbaum, A.; Strong, L. C.; and Gardner, W. U. Influence of Methylcholanthrene on Age Incidence of Leukemia in Several Strains of Mice. Proc. Soc. Exper. Biol. & Med., **45**:287–89, 1940.
11. McEndy, D. P.; Boon, M. C.; and Furth, J. Induction of Leukemia in Mice by Methylcholanthrene and X-Ray. J. Nat. Cancer Inst., **3**:227–47, 1942.
12. Murphy, J. B., and Sturm, E. The Transmission of an Induced Lymphatic Leukemia and Lymphosarcoma in the Rat. Cancer Research, **1**:379–83, 1941.
13. Oberling, C. H.; Guérin, M.; and Guérin, P. Leucémies spontanées et transplantables du rat. Bull. Assoc. franç. l'étude cancer, **28**:214–42, 1939.
14. Rask-Nielsen, R. Experimental Studies on a Transplantable Aleukemic Myelomatosis in White Rats. Acta path. et microbiol. Scandinav., **15**:285–300, 1938.
15. Ratcliffe, H. L. Spontaneous Tumors in Two Colonies of Rats of the Wistar Institute of Anatomy and Biology. Am. J. Path., **16**:237–54, 1940.
16. Roffo, A. H. Carcinogenic Value of Oxidated Oils. Am J. Digest. Dis., **13**:33–38, 1946.
17. Shay, H.; Aegerter, E. A.; Gruenstein, M.; and Komarov, S. A. Development of Adenocarcinoma of the Breast in the Wistar Rat Following the Gastric Instillation of Methylcholanthrene. J. Nat. Cancer Inst., **10**: 255–66, 1949.
18. Shay, H., and Gruenstein, M. A Simple and Safe Method for the Gastric Instillation of Fluids in the Rat. J. Lab & Clin. Med., **31**:1384–86, 1946.
19. Shay, H.; Weinhouse, S.; Gruenstein, M.; Marx, H. E.; and Friedman, B. The Development of Malignant Lymphoma in Some of the Young Rats Suckled by Mothers Receiving Methylcholanthrene by Stomach Tube Only during Lactation Period (Preliminary Report). Cancer, **3**:891–95, 1950.
20. Stewart, H. L., and Lorenz, E. Induction of Adenocarcinoma of the Pyloric Stomach in Mice by Methylcholanthrene. J. Nat. Cancer Inst., **2**:193–96, 1941.
21. Storti, E., and Storti, R. Modifications morphologiques du sang et des organes hématopoïétiques provoquées chez le rat blanc par des injections intra-médullaires de 1:2 benzopyrène. Sang, **11**:749–53, 1937.
22. Thomsen, O., and Engelbreth-Holm, J. Experimentelles Hervorrufen von leukotischen Zuständen bei Hühnern. Acta path. et microbiol. Scandinav., **8**:121–84, 1931.
23. Wilens, S. L., and Sproul, E. E. Spontaneous Leukemia and Chlorleukemia in the Rat. Am. J. Path., **12**:249–57, 1936.
24. Zucker, T. F.; Berg, B. N.; and Zucker, L. M. Nutritional Effects on the Gastric Mucosa of the Rat. I. Lesions of the Antrum. J. Nutrition, **30**:301–17, 1945.

Combinations of Chemical Compounds in Experimental Cancer Therapy*

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The ability of microbial cells to adapt themselves to toxic agents leads to the development of "drug fastness" in anti-bacterial chemotherapy. Experimental evidence indicates that two mechanisms may be important in the resistance of bacteria to chemical agents. Bacterial mutants may arise which are no longer dependent upon the metabolic pathway which was blocked in the parent strain by the anti-bacterial agent. Adaptations of metabolism may occur so that an alternative pathway is developed which is not affected by the drug. In the chemotherapy of infectious diseases, significant advances have been made by the discovery of more potent anti-bacterial chemical compounds and by the judicious use of combinations of effective drugs. Combination therapy is effective against selected microbial diseases, presumably because the drugs used may attack the metabolic function of the microbial cell simultaneously at different points.

In experimental and clinical cancer chemotherapy no chemical compound has yet been developed which destroys neoplastic cells effectively without serious toxicity to normal cells. In view of the well recognized qualitative similarities between normal and malignant cells, it seems unlikely that a single chemical agent will be found which will selectively kill tumor cells. It is for this reason that the use of combinations of chemical compounds in the treatment of tumors in experimental animals has been explored in our laboratory. Limited trial of this approach has been made. Mitchell (13) has reported that the combination of parenterally administered tetra-sodium 2-methyl-1:4-naphthohydroquinone diphosphate (synkavit) and irradiation treatment has slightly prolonged the mean survival time of patients with inoperable carcinoma of the bronchus (13), but this has yet to be substantiated and has even been questioned (6). Skipper (16) has observed a possible anti-leukemic synergism between urethan and nitrogen mustard (HN_2) against a strain of mouse leukemia. Emerson, Wurtz, and Zanetti (5) have reported the rapid regression of lymphosarcoma transplants in mice receiving cortisone in conjunction with a riboflavin-deficient diet, and methyl testosterone or cortisone administered to pyridoxine-deficient rats increases the tumor inhibition of a rat lymphosarcoma produced by either steroid alone (2).

Reports of the effect of 5-amino-7-hydroxy-1*H*-*v*-triazolo (*d*) pyrimidine (guanazolo, 8-azaguanine) on a variety of neoplasms in experimental animals (3, 7, 9, 11, 12, 17, 18, 20) and of its mechanism of action (15, 25) have appeared with increasing frequency during the past year. In the present report, preliminary experiments will be summarized which indicate that the administration of certain chemical agents together with 8-azaguanine can lead to a greater inhibition of tumor growth than is obtained with 8-azaguanine alone.

MATERIALS AND METHODS

The carcinostatic action of guanazolo upon the 755 tumor, a transplantable mammary adenocarcinoma in C57 black mice, has been previously established and recorded in this laboratory (7). In the experiments to be reported, combinations of chemical compounds were assayed for their inhibitory effect upon the growth of this mouse tumor, and 8-azaguanine always constituted one component of the combination under trial. The effectiveness of combinations has been measured by the comparison of results obtained following the intraperitoneal administration of 8-azaguanine alone with those observed following combination therapy.

The tumor was transplanted unilaterally in the axillary region by the usual trocar method. The mice were allowed a stock diet of Rockland pellets and water *ad libitum*. Therapy was initiated at

* This work was aided by a grant from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council and by the Ellen B. Green Memorial Fund Gift for Cancer Research.

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Received for publication August 7, 1950.

various intervals after transplantation as indicated in the tabular summaries, the experiments were terminated by killing the animals, and the tumors were dissected out and weighed to the nearest milligram.

The chemical compounds[1] were dissolved as follows: 8-azaguanine in 0.1 N NaOH as previously described (7); desoxypyridoxine and vitamin B_{12} in isotonic saline; pteroylglutamic acid and 7-methyl folic acid in 2 per cent sodium bicarbonate. Control animals received injections of comparable volumes of the appropriate vehicle.

RESULTS

Desoxypyridoxine.—The results of the administration of the pyridoxine analog, desoxypyridoxine, are summarized in Table 1. In this table and in all subsequent ones, the pertinent information on the number of animals, their mortality, the number of tumors, the duration of the experiment, the time of administration of the drugs, dosage schedules, mean tumor weights, and the standard deviation of the mean is presented so that the protocol of each experiment can be duplicated. In comparing the difference between two mean tumor weights (m_1 and m_2), the results have been considered beyond chance variation when

$$\frac{m_1 - m_2}{\sqrt{\sigma m_1^2 + \sigma m_2^2}} = 2.5$$

or greater. The average body weight of all groups of animals is recorded at the beginning and the end of each experiment in order to assess the possible nonspecific contribution of weight loss on tumor inhibition.

As can be seen from Table 1, desoxypyridoxine, given alone, significantly inhibited tumor growth in two experiments (Exp. Nos. 74 and 79), as compared with untreated controls, and significantly failed to modify tumor growth in two other experiments (Exp. Nos. 67 and 92). It is to be noted that in no instance was the desoxypyridoxine-induced tumor inhibition as great as that consistently observed with 8-azaguanine. In five out of seven experiments the combination of 8-azaguanine and desoxypyridoxine inhibited tumor growth to a significantly greater extent than the purine analog alone. In the two experiments (Exp. Nos. 74 and 106) in which the difference in mean tumor weights was not significant, the trend was in the same direction as in the other experiments.

[1] We wish to express our appreciation to Dr. J. M. Ruegsegger of the Lederle Laboratories, Pearl River, N.Y., for the 8-azaguanine, to Drs. A. Gibson and H. Robinson of Merck & Co., Inc., Rahway, N.J., for the desoxypyridoxine and vitamin B_{12}; and to Dr. G. Martin of the National Drug Co., Philadelphia, Pa., for the 7-methyl folic acid.

The dosage levels employed ranged from 200 to 175 mg/kg initially, dropping during the course of treatment to 175–150 mg/kg of body weight. When 150 to 135 mg desoxypyridoxine/kg body weight were employed initially in combination with 8-azaguanine, the results of such experiments, although showing a definite trend in the direction of augmented inhibition of tumor growth, were statistically insignificant. It would appear that dosage levels in the neighborhood of 150 mg/kg would be in the threshold range of a desoxypyridoxine-azaguanine combination effect.

With the exception of Experiment 85, the weight loss produced by this combination falls into a range which, in our experience, has not affected tumor growth. Experiment 85 is nevertheless considered a valid experiment, because the weight loss is of the same degree in both the azaguanine-treated and in the desoxypyridoxine–azaguanine-treated animals.

Folic acid.—Tables 2 and 3 illustrate the effects of the administration of pteroylglutamic acid 1 hour before that of 8-azaguanine on tumor growth in male and female mice. It can be noted that in neither series of experiments did pteroylglutamic acid have an inhibitory effect on the tumor when compared with untreated controls. As can be seen from the results, a difference in the effect of the combination of 8-azaguanine and folic acid on tumor inhibition was detectable, depending upon the sex of the host. In four out of five experiments on male mice, significant inhibition of tumor growth followed the administration of the combination, whereas in none of the three experiments on female mice was the difference between the mean tumor weights of the combination-treated and 8-azaguanine-treated animals significant. The observed variation in response in the two sexes is not understood.

7-Methyl folic acid.—The effects on tumor growth of the simultaneous administration of 7-methyl folic acid and 8-azaguanine, compared with 8-azaguanine therapy alone, are summarized in Table 4. It is to be noted that 7-methyl folic acid alone failed to inhibit tumor growth, whereas the combination of the folic acid derivative and the purine analog decreased the size of tumors to a significantly greater extent than did 8-azaguanine alone in all experiments except No. 118. It is also of interest that, unlike the observations with folic acid previously described, the inhibitory effect of 7-methyl folic acid and 8-azaguanine was noted whether the tumor-bearing hosts were male or female.

Vitamin B_{12}.—The results of combination therapy with Vitamin B_{12} and 8-azaguanine are sum-

marized in Table 5. Significant tumor inhibition by the combination is far less consistent than in the previously recorded experiments, and B_{12} alone has no effect.

In the nine experiments conducted, the combination of B_{12} and 8-azaguanine significantly depressed tumor growth in three instances (Exp. Nos. 67, 91, and 95). In one experiment (Exp. No. 74) the tumors of combination-treated animals were significantly larger than those in the 8-azaguanine controls, and in the remaining observations no significant difference between 8-azaguanine and 8-azaguanine plus B_{12} treatment could be determined. Table 5 indicates that the dosage of vitamin B_{12} varied from 25 μg to 2,000 μg/kg body weight and that the B_{12} was injected at different time-intervals in relation to the 8-azaguanine. The results of experiments 91, 95, and 108 suggest the possibility that the dosage and the time of administration of the B_{12} may be important in obtaining consistent tumor inhibition when combined with 8-azaguanine.

Histology.—In all the experiments, histological sections of tumors from the experimental and control animals failed to show distinctive morphological changes which distinguished the groups.

DISCUSSION

The basis for the selection of the chemical compounds used in combination with 8-azaguanine in these experiments rested on two hypotheses.

Qualitative differences in the biochemical patterns of normal and neoplastic cells have not yet been described; however, many quantitative dif-

TABLE 1

EFFECT OF DESOXYPYRIDOXINE AND 8-AZAGUANINE ON THE 755 TUMOR IN C57 MICE

Exp. no.	Group	No. animals dead/alive	No. tumors	Duration tumor growth (days)	Day therapy begun after transplantation	Daily dosage (mg/kg) DP*	Daily dosage (mg/kg) 8-AG†	Mean tumor weight ± standard deviation of mean (mg.)	Av. body weight (gm.) Beginning	Av. body weight (gm.) End
74	Controls	1/10	9	18	8			888± 51	22.3	23.3
	Desoxypyridoxine	3/10	6	18	8	175		486±151	22.4	22.0
♂	8-Azaguanine	0/10	10	18	8		50	51± 7	21.4	21.1
	8-Azaguanine +Desoxypyridoxine	5/10	5	18	8	175	50	36± 10	22.4	20.0
79	Controls	0/30	26	18	1			284± 23	18.8	18.2
♀	Desoxypyridoxine	9/30	16	18	1	‡		155± 43	19.5	19.0
92	Controls	4/20	16	23	13			1110±172	24.0	25.6
♂	Desoxypyridoxine	1/20	19	23	13	§		1135±117	23.4	22.5
	8-Azaguanine	0/30	28	23	13		50	324± 32	24.1	22.9
	8-Azaguanine +Desoxypyridoxine	5/30	22	23	13	§	50	209± 25	23.0	20.8
	Sacrificed controls	0/20	20	13				181± 24	23.5	
67	Controls	0/20	19	16	5			587± 61	21.5	22.1
♂	Desoxypyridoxine	0/10	10	16	5	175		459± 76	22.0	21.6
	8-Azaguanine	0/18	18	16	5		50	82± 11	22.2	22.5
	8-Azaguanine +Desoxypyridoxine	1/10	7	16	5	175	50	29± 4	21.7	20.3
95	Controls	0/10	10	20	8			1932±147	22.9	23.8
♂	8-Azaguanine	0/10	10	20	8		50	200± 28	20.6	19.9
	8-Azaguanine +Desoxypyridoxine	0/10	10	20	8	‖	50	103± 18	20.1	19.2
85	8-Azaguanine	6/30	24	25	14		50	959± 57	23.1	19.5
♂	8-Azaguanine +Desoxypyridoxine	5/30	25	25	14	175	50	512± 58	22.2	18.8
106	Controls	1/10	9	20	7			1420±251	21.1	20.9
♂	8-Azaguanine	1/10	9	20	7		50	220± 45	20.5	19.9
	8-Azaguanine +Desoxypyridoxine	0/10	10	20	7	175	50	106± 16	20.5	17.9
114	8-Azaguanine	0/10	10	24	7		50	398± 66	18.7	18.3
♂	8-Azaguanine +Desoxypyridoxine	0/10	10	24	7	175	50	195± 27	18.1	17.0

* DP = Desoxypyridoxine.

† 8-AG = 8-Azaguanine.

‡ 200 (×4), then 175 (×11).

§ 200 (×2), then 175 (×7).

‖ 175 (×4), then 150 (×6).

ferences in the two types of cells have been recorded (10). Of particular pertinence to the present discussion are the quantitative assays of the concentrations of members of the Vitamin B complex in normal and tumor tissue (2, 23). Since, for example, it has been demonstrated that pyridoxine is in lower concentration in certain tumors than in the analogous normal tissue, it was considered possible to inhibit the function of this vitamin completely, by means of an antagonist in cancer tissue, while minimally depressing the metabolic activities of the vitamin in normal tissues. This is a reasonable hypothesis, since metabolite inhibition by an anti-metabolite depends to a large degree upon the concentration of the metabolite. Ackermann and Potter have recently presented evidence which indicates that the same holds true for enzymes (1).

On the basis of the above hypothesis, a series of vitamin analogs were selected for chemotherapeutic trial in combination with 8-azaguanine on the 755 tumor. At this time, desoxypyridoxine is the only analog with which preliminary observations have been completed. The mechanism of action, whereby desoxypyridoxine in combination with 8-azaguanine inhibits tumor growth to a greater extent than is observed following treatment with either chemical compound alone, is not known. In support of the concept presented is the fact that desoxypyridoxine has been shown to be a pyridoxine antagonist (4, 21); however, a number

TABLE 2

EFFECT OF 8-AZAGUANINE AND PTEROYLGLUTAMIC ACID ON THE 755 TUMOR IN ♂ C57 MICE

Exp. no.	Group	No. animals dead/alive	No. tumors	Duration tumor growth (days)	Day therapy begun after transplantation PGA*	8-AG†	Daily dosage (mg/kg) PGA	8-AG	Mean tumor weight ± standard deviation of mean (mg.)	Av. body weight (gm.) Beginning	End
84	8-Azaguanine	1/30	28	34		14		50	830± 69	23.0	22.5
	PGA+8-AG	1/30	29	34	13	14	50	50	441± 31	23.8	22.0
105	8-Azaguanine	0/20	20	26		12		50	1159±132	20.2	20.2
	PGA+8-AG	0/20	19	26	11	12	50	50	820± 69	20.9	20.1
76A	Controls	1/10	9	25					1770±320	23.7	23.8
	PGA	4/10	6	25	7		50		2146±431	21.6	24.7
	8-Azaguanine	0/10	10	32		8		50	522± 72	21.9	21.8
	PGA+8-AG	0/10	10	32	7	8	50	50	321± 34	23.9	22.2
76B	Controls	1/10	9	25					1770±320	23.7	23.8
	PGA	5/10	5	25	7		37.5		1796±338	22.1	24.0
	8-Azaguanine	0/10	10	32		8		50	522± 72	21.9	21.8
	PGA+8-AG	0/10	10	32	7	8	37.5	50	262± 37	23.7	23.1
	Sacrificed controls	0/10	10	8					36± 11	24.6	
72	8-Azaguanine	1/10	9	22		13		50	129±11.1	17.7	18.7
	PGA+8-AG	2/10	8	22	12	13	37.5	50	49± 9.4	15.5	16.6

* PGA = pteroylglutamic acid.
† 8-AG = 8-azaguanine.

TABLE 3

EFFECT OF 8-AZAGUANINE AND PTEROYLGLUTAMIC ACID ON THE 755 TUMOR IN ♀ C57 MICE

Exp. no.	Group	No. animals dead/alive	No. tumors	Duration tumor growth (days)	Day therapy begun after transplantation PGA	8-AG†	Daily dosage (mg/kg) PGA*	8-AG	Mean tumor weight ± standard deviation of mean (mg.)	Av. body weight (gm.) Beginning	End
83	8-Azaguanine	1/29	28	34		14		50	905± 96.5	20.6	20.0
	PGA+8-AG	1/30	29	34	13	14	50	50	790± 61.5	20.9	21.5
104	8-Azaguanine	2/20	18	26		12		50	790± 84	18.2	18.5
	PGA+8-AG	0/20	20	26	11	12	50	50	590± 64	18.7	19.1
75	Controls	0/10	10	18					503±122	19.6	19.3
	PGA	0/10	10	18	7		37.5		847± 78	19.7	20.5
	8-Azaguanine	0/10	10	18		8		50	67± 14.1	18.9	18.3
	PGA+8-AG	1/10	9	18	7	8	37.5	50	30± 6.7	18.2	18.7

* PGA = pteroylglutamic acid.
† 8-AG = 8-azaguanine.

TABLE 4

Effect of 7-Methyl Folic Acid and 8-Azaguanine on the 755 Tumor in C57 Mice

Exp. no.	Group	No. animals dead/alive	No tumors	Duration tumor growth (days)	Day therapy begun after transplantation 7-Methyl PGA	8-AG	Daily dosage (mg/kg) 7-Methyl PGA	8-AG	Mean tumor weight ± standard deviation of mean (mg.)	Av. body weight (gm.) Beginning	End
91	Controls	1/10	9	19					788±111	17.7	18.4
♀	8-Azaguanine	0/10	10	19		7		50	278± 58	17.9	17.6
	7-Methyl PGA	0/10	10	19	7		*		693±147	18.2	17.3
	7-Methyl PGA +8-Azaguanine	0/10	10	19	7	7	*	50	48± 4	18.3	15.8
95	Controls	0/10	10	20					1125±147	22.9	23.8
♂	8-Azaguanine	0/10	10	20		8		50	200± 28	20.6	19.9
	7-Methyl PGA	0/10	10	20	8		†		1460± 18	22.6	23.1
	7-Methyl PGA +8-Azaguanine	1/10	9	20	8	8	†	50	67± 9	19.3	18.9
107	8-Azaguanine	1/10	8	22		8		50	446± 89	18.2	18.7
♀	7-Methyl PGA +8-Azaguanine	1/10	8	22	8	8	‡	50	169± 48	17.2	17.0
108	8-Azaguanine	0/10	10	22	8	8		50	386± 47	19.2	19.3
♂	7-Methyl PGA +8-Azaguanine	0/10	10	22	8	8	‡	50	220± 39	19.4	19.3
118	8-Azaguanine	2/10	8	33		7		50	785±113	17.1	18.3
♀	7-Methyl PGA +8-Azaguanine	0/15	15	33	7	7	25	50	1117±121	17.3	18.6

* 50 (×4), then 25 (×4).
† 35 (×4), then 25 (×6).
‡ 30 (×5), then 25 (×5).

TABLE 5

Effect of Vitamin B_{12} and 8-Azaguanine on the 755 Tumor in C57 Mice

Exp. no.	Group	No. animals dead/alive	No. tumors	Duration tumor growth (days)	Day therapy begun after transplantation B_{12}	8-AG	Daily dosage μgm/kg B_{12}	mg/kg 8-AG	Mean tumor weight ± standard deviation of mean (mg.)	Av. body weight (gm.) Beginning	End
67	Controls	0/20	19	16					587± 61	22.4	22.3
♂	B_{12}	0/10	10	16	5		*		618±116	23.3	22.3
	8-Azaguanine	0/18	18	16		5		50	82± 11	22.2	22.5
	B_{12}+8-AG	1/10	9	16	5	5	*	50	37± 7	23.3	22.6
86	8-Azaguanine	6/30	24	28		15		50	1055± 99	21.6	20.6
♂	B_{12}+8-AG	0/30	28	28	14	15	200	50	1205± 75	21.9	19.9
74	Controls	1/10	9	18					888± 51	22.3	23.3
♂	B_{12}	0/10	10	18	8		1,250		630± 95	21.2	22.1
	8-Azaguanine	0/10	10	18		8		50	51± 7	21.4	21.1
	B_{12}+8-AG	0/10	10	18	8	8	1,250	50	86± 9	20.7	20.1
87	8-Azaguanine	5/30	25	28		15		50	1404± 98	21.4	20.4
♂	B_{12}+8-AG	8/30	22	28	14	15	2,000	50	1149± 93	21.5	20.0
91	Controls	1/10	9	19					788±111	17.7	18.4
♀	8-Azaguanine	0/10	10	19		7		50	278± 58	17.9	17.6
	B_{12}+8-AG	0/10	10	19	4	7	1,250*	50	85± 14	18.5	19.2
95	Controls	0/10	10	20					1125±147	22.9	23.8
♀	8-Azaguanine	0/10	10	20		8		50	200± 28	20.6	19.9
	B_{12}+8-AG	0/10	10	20	5	8	† ‡	50	93± 15	20.3	20.4
108A	8-Azaguanine	0/10	10	22		8		50	386± 47	19.2	19.3
♂	B_{12}+8-AG	0/10	10	22	6	8	1,000*	50	249± 42	14.1	19.3
108B	8-Azaguanine	0/10	10	22		8		50	386± 47	19.2	19.3
♂	B_{12}+8-AG	0/10	10	22	6	8	500*	50	285± 30	19.7	19.3
118	8-Azaguanine	2/10	8	33		7		50	785±113	17.1	18.3
♀	B_{12}+8-AG	1/15	14	33	7	7	1,000*	50	983±112	17.9	19.5

* 25 (×5), then 250 (×5).
† = B_{12} dose given ½ hour before 8-azaguanine dose.
‡ 1250 (×6), then 300 (×7).

of other possibilities must be considered and studied before accepting the mechanism of action as due to competitive inhibition of pyridoxine function.

Microbiological studies with pteroylglutamic acid and vitamin B_{12} suggest their importance in nucleic acid metabolism (14, 24). Since other studies from this laboratory have shown an effect of 8-azaguanine on mitotic activity in tumor cells (15), it was considered possible that folic acid and vitamin B_{12} might prevent the carcinostatic effect of the guanine analog. As was shown in the "Results," these vitamins not only failed to reverse the 8-azaguanine action but, rather, led to a greater inhibition of tumor growth. 7-Methyl folic acid, a relatively weak antagonist of pteroylglutamic acid, was selected primarily because of its structural similarity to folic acid.

The mechanism by which pteroylglutamic acid, its 7-methyl analog, or vitamin B_{12}, when combined with 8-azaguanine, lead to greater tumor inhibition than that observed following 8-azaguanine alone is not known. A possible explanation for the folic acid effect is that one of its degradation products, 2-amino-4-hydroxy-6-formylpteridine, inhibits a liver enzyme system *in vitro* which deaminates 8-azaguanine.[2] The resulting 2,4-dihydroxytriazolopyrimidine is inactive as a carcinostatic agent (8). This finding may be comparable to that of Williams and Elvehjem that "the metabolism of guanine (presumably via guanase to xanthine . . .)" is decreased by folic acid (22). Whether this possible explanation of the mechanism involved in the folic acid observations is pertinent to the studies with 7-methyl folic acid or vitamin B_{12} is not known.

The mechanism of action of the combinations utilized in these experiments is of particular interest, as it may provide some insight into the metabolism of the tumor cells. The most obvious explanation for the observed enhancement of carcinostasis would be that the administration of two chemical compounds caused greater toxicity to the host than either drug alone and that the tumor inhibition was merely a reflection of over-all toxicity. The evidence does not support this mechanism, however. It can be seen from the results that in the great majority of instances there was either no weight loss or a minimal loss in the animals, and, therefore, on the reasonable assumption that loss of body weight constitutes a measure of general toxicity, there was no significant deleterious effect of the combinations on the host. Furthermore, initial trials with a number of other vitamin analogs in combination with 8-azaguanine have failed to show an enhancement of tumor inhibition, thus lending circumstantial support to our conviction that the reported results are specific rather than nonspecific.

[2] A. Graff, J. Kream, and A. Gellhorn, unpublished results.

SUMMARY AND CONCLUSIONS

The effect of combinations of chemical compounds in cancer chemotherapy has been investigated. By the use of the guanine analog, 5-amino-7-hydroxy-1*H*-*v*-triazolo (*d*) pyrimidine, together with either desoxypyridoxine, pteroylglutamic acid, 7-methyl pteroylglutamic acid, or vitamin B_{12}, it has been shown that the growth of a transplantable carcinoma of the breast in mice can be inhibited to a greater extent by a combination than by any one of the drugs alone. Evidence has been presented to show that the combinations are not acting by causing host toxicity, for the observed effects are associated with minimal to no weight loss. The possible mechanisms of action are discussed.

It is recognized that in no instance did a combination eradicate the tumor being treated; however, the possibility of utilizing more effective agents in combination or of increasing the number of active chemical compounds in combination offers an attractive approach to achieve this end.

ACKNOWLEDGMENTS

The authors wish to express their appreciation to Misses Ruth Krueger, Alice Kells, Gertrude Grille, and Annette Hartig for their cooperation and technical assistance.

REFERENCES

1. Ackermann, W. W., and Potter, V. R. Enzyme Inhibition in Relation to Chemotherapy. Proc. Soc. Exper. Biol. & Med., **72**:1–9, 1949.
2. Ballantyne, R. M., and McHenry, E. W. Vitamin B_6 and Biotin in Human Cancer Tissue. Cancer Research, **9**:689–91, 1949.
3. Burchenal, J. H.; Johnston, S. F.; Burchenal, J. R.; Kushida, N. M.; Robinson, E.; and Stock, C. C. Chemotherapy of Leukemia. IV. Effect of Folic Acid Derivatives on Transplanted Mouse Leukemia. Proc. Soc. Exper. Biol. & Med., **71**:381–87, 1949.
4. De Renzo, E. C., and Cerecedo, L. R. Effects of Desoxy- and Methoxypyridoxine in the Mouse. Proc. Soc. Exper. Biol. & Med., **73**:356–59, 1950.
5. Emerson, G. A.; Wurtz, E.; and Zanetti, M. E. Regression of Lymphosarcoma Transplants Following the Administration of Cortisone to Riboflavin-deficient Mice. Fed. Proc., **9**:357, 1950.
6. Gellhorn, A., and Gagliano, T. The Effect of Tetra-Sodium 2-Methyl 1:4-Naphthohydroquinone Diphosphate on a Variety of Tumors in Experimental Animals. Brit. J. Cancer, **4**:103–8, 1950.
7. Gellhorn, A.; Engelman, M.; Shapiro, D. M.; Graff, S.; and Gillespie, H. The Effect of Guanazolo on a Variety of Neoplasms in Experimental Animals. Cancer Research, **10**:170–77, 1950.

8. GELLHORN, A. Purines and Pyrimidines in Experimental Cancer Chemotherapy. Proc. Symp. Chem. Biol. Coordination Center, 1950.
9. GREENBERG, D. M.; IRISH, A. J.; and GAL, E. M. Effect on Metabolite Analogs on Growth of Transplantable Tumors. Cancer Research, **10**:221–22, 1950.
10. GREENSTEIN, J. P. Biochemistry of Cancer. New York: Academic Press, Inc., 1947.
11. KIDDER, G. W.; DEWEY, V. C.; PARKS, R. E., JR.; and WOODSIDE, G. L. Purine Metabolism in Tetrahymena and Its Relation to Malignant Cells in Mice. Science, **109**:511–14, 1949.
12. LAW, L. W. Studies on the Effects of a Guanine Analog on Acute Lymphoid Leukemias of Mice. Cancer Research, **10**:186–90, 1950.
13. MITCHELL, J. S. Clinical Trials of Tetra-Sodium 2-Methyl-1:4-Naphthohydroquinone Diphosphate in Conjunction with X-Ray Therapy. Brit. J. Cancer, **2**:351–59, 1948.
14. ROGERS, L. L., and SHIVE, W. Biochemical Transformations as Determined by Competitive Analogue-Metabolite Growth Inhibitors. VII. Relationship of Purines and Thymine to Folic Acid, J. Biol. Chem., **172**:751–58, 1948.
15. SHAPIRO, D. M.; WEISS, R.; and GELLHORN, A. The Effect of 8-Azaguanine on Mitosis in Normal and Neoplastic Tissues. Cancer, **3**:896–902, 1950.
16. SKIPPER, H. E. Carbamates in the Chemotherapy of Leukemia. V. Observation of a Possible Antileukemic Synergism between Urethane and Methylbis (β-chlorethyl) Amine. Cancer, **2**:475–79, 1949.
17. SOKOLOFF, B.; REDD, J. B.; and DUTCHER, R. Metabolite Antagonists in Experimental Cancer: Guanazolo. Fed. Proc., **9**:344, 1950.
18. STOCK, C. C.; CAVALIERI, L. F.; HITCHINGS, G. H.; and BUCKLEY, S. M. A Test of Triazolopyrimidines on Mouse Sarcoma 180. Proc. Soc. Exper. Biol. & Med., **72**:565–67 1949.
19. STOERK, H. C. The Retarding Effect of Methyl Testosterone and of Cortisone upon the Growth of Lymphosarcoma Implants in Pyridoxine-Deficient Rats. Proc. Soc. Exper. Biol. & Med. (in press).
20. SUGIURA, K.; HITCHINGS, G. H.; CAVALIERI, L. F.; and STOCK, C. C. The Effect of 8-Azaguanine on the Growth of Carcinoma, Sarcoma, Osteogenic Sarcoma, Lymphosarcoma and Melanoma in Animals. Cancer Research, **10**: 178–85, 1950.
21. UMBREIT, W. W., and WADDELL, J. G. Mode of Action of Desoxypyridoxine. Proc. Soc. Exper. Biol. & Med., **70**: 293–99, 1949.
22. WILLIAMS, J. N., JR., and ELVEHJEM, C. A. Some Relationships of Folic Acid to Structurally Similar Metabolites. Proc. Soc. Exper. Biol. & Med., **71**:303–5, 1949.
23. WILLIAMS, R. J. B Vitamins and Cancer. A.A.A.S. Research Conference on Cancer, p. 259, 1944.
24. WRIGHT, M. H. Thymidine and Vitamin B_{12}. Science, **110**:257–58, 1949.
25. YOUNGNER, J. S., and SALK, J. E. Effect of a Guanine Analog (Guanazolo) on Viral Multiplication and on Growth of the Chick Embryo. Cancer Research, **10**:250–51, 1950.

Test of Correlation between the Pink-Eye Gene and Susceptibility to Induced Fibrosarcoma in Mice*

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Mice of inbred strains with pink eyes (I, JK) show a lower incidence of methylcholanthrene-induced fibrosarcomas than do mice with dark eyes. Mice of thirteen other inbred strains, similarly treated with methylcholanthrene (1 mg. injected subcutaneously in 0.1 cc. of sesame oil at 60 days of age), showed a higher incidence of induced tumors. Furthermore, the "screening" of the fifteen strains revealed correlations between five genetic markers and fibrosarcoma susceptibility, as follows: (*a*) brown versus black, (*b*) nonagouti versus agouti, (*c*) piebald versus self, (*d*) pink eye versus dark eye, and (*e*) female versus male (7). Mice with multiple dominant genetic complexes, such as C3H, with dark eye, intense, black, agouti, and self show the highest incidence of chemically induced fibrosarcomas. Mice with multiple recessive complexes, such as the I strain with five recessive genes, pink eye, dilute, brown, nonagouti, and piebald have shown the greatest resistance to fibrosarcomas. Between the two extremes are mice of the other thirteen inbred strains with various combinations of genetic markers and susceptibilities to fibrosarcomas.

Since mice of the C3H strain possess wild type characters, these facts cited above may mean that mice with the fewest mutants have the highest susceptibility to induced fibrosarcomas.

The F_2 pink-eye segregates of a cross between dark- and pink-eyed mice (Brs × I strains) showed approximately 15 per cent fewer induced tumors at 325 days than their dark-eyed sibs. Thus, of 353 F_2 pink-eyed mice injected with methylcholanthrene, 151, or 42.7 per cent, developed fibrosarcomas by 325 days; whereas of 1,204 F_2 dark-eyed mice similarly treated, 697, or 57.9 per cent, developed fibrosarcomas within the same period. The experiment was terminated at 325 days following the injection of the methylcholanthrene. The χ^2 determination of this difference is 24.15, which is significant. This evidence indicated a correlation between pink eye and a lower fibrosarcoma susceptibility.

The present experiment continues observations on the relationship between the gene for pink eye and the rate at which fibrosarcoma appears in mice following the subcutaneous injection of methylcholanthrene.

MATERIALS AND METHODS

Dark-eyed, brown, nonagouti, long-eared, female mouse No. 133,716 of the F_3 generation of the NH descent (5) was outcrossed to a male of the pink-eyed, brown, nonagouti, short-eared, JK strain. This mating produced three litters of sixteen young, all of which were dark-eyed, brown, nonagouti, and long-eared. Male mouse No. 134,442 (dark-eyed, brown, nonagouti, long-eared) of the NH strain was mated to a female mouse of the JK strain, who produced four litters of 21 mice, all of which were dark-eyed, brown, nonagouti, and long-eared. Thus, it was concluded that each of these F_3 mice was homozygous for dark eyes, and they were then mated together and the progeny continued for the Br (dark-eyed, brown, nonagouti) descent. One mg. of methylcholanthrene, dissolved in 0.1 cc. of sesame oil, was injected subcutaneously into both parents of each generation at 60 days of life. During the ensuing ten generations of direct descent, 264 mice were produced, all of which were dark-eyed, brown, and nonagouti. In the collateral lines, dark-eyed, brown, and nonagouti mice were obtained in excess of ten thousand. The mating of mouse No. 219,009 female and No. 219,010 male of the F_{13} generation produced 42 young, 38 of which were dark-eyed and 4 pink-eyed. One of these pink-eyed mutants was outcrossed to a mouse of the pink-eyed, brown, nonagouti, short-eared JK strain and produced seventeen young, all of which were pink-eyed, brown, nonagouti, and long-eared. It was concluded that a new pink-eye mutation had occurred at the pink-eye or "p"

* This experiment has been made possible by grants from The Anna Fuller Fund, The Jane Coffin Childs Memorial Fund for Medical Research, and the Public Health Service, acting through the National Advisory Cancer Council.

Received for publication August 18, 1950.

locus. Thus, pink eye had occurred as a mutation somewhere in the preceding descent (between the F_4 and F_{12}).

The Brp subline of the NH descent consists of pink-eyed and dark-eyed mice which were descended from one of the original pink-eyed mutants mated to a dark-eyed individual of the NH line. The origin of the Brp descent is given in Chart 1. The pink-eyed mutant ("Brp" subline) has been continued for twelve generations (F_{14}–F_{25}) by mating a pink-eyed individual to a sib heterozygous for dark eye. The "Brp" descent has produced 641 mice; 323 dark-eyed and 318 pink-eyed (expectation of 1:1 ratio would be 320.5 dark-eyed:320.5 pink-eyed). The sex ratio has been 349 females to 292 males (54.5 per cent females:45.5 per cent males).

One mg. of methylcholanthrene dissolved in 0.1 cc. of sesame oil was injected in the right flank, when the mice were 60 days old. The mice were examined weekly for the appearance of tumors at the site of injection and were killed as soon as the tumors became firm and progressively increased in size between two periods of observations. All mice dying or killed during the experiment were examined at autopsy, and lesions were fixed in Bouin's fixative and stained with hematoxylin and eosin.

RESULTS

Mice of the "Brp" lineage injected with 1 mg. of methylcholanthrene gave rise to three types of tumors: (*a*) fibrosarcoma, arising at the site of the injection of methylcholanthrene; (*b*) adenomas of the lungs, and (*c*) a gastric lesion involving the glandular mucosa near the pylorus, similar to lesions arising in mice of the Brs descent (8). The gastric lesion has also been discussed by McPeak and Warren (3), Smith and Strong (4), Kaplan (2), and Bagshaw (1). The tumors were similar to those appearing in mice of the ancestral Br subline. The incidence of the three types of tumors in Brp mice, together with comparative data for the Br descent, are given in Table 1.

The percentage incidence of the three types of tumors (total tumors) in the mutant subline is approximately the same as that for the mice of the parental Br subline (79.9 per cent of total tumors

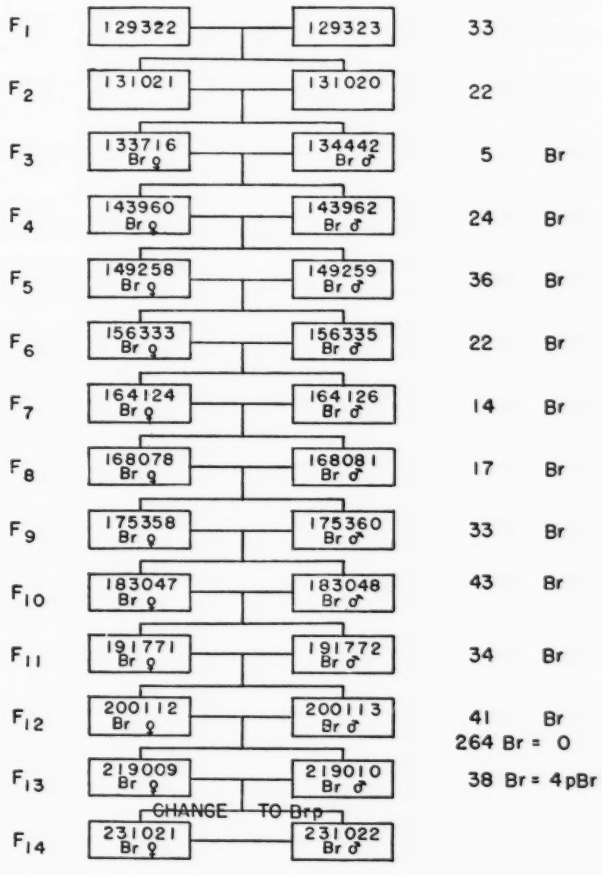

CHART 1.—Lineal descent of a single pair of mice of a cross between the CBAN and JK mice. Parentage for the first fourteen generations are given. The number of progeny produced in each mating is given on the right. All mice from the F_2 generation onward were injected with methylcholanthrene. Pink-eye mutants (pBr) appeared for the first time in the F_{13} generation.

TABLE 1

PERCENTAGE OF TUMORS IN MICE*

Mice of the Br subline and of the two color classes of the pink-eye mu ant subline Brp. The sexes are separate, and the three types of lesions and total tumors are given separately.

tock	Color	Sex	No. inj.	Total tumors	Per cent total tumors	Local tumors	Per cent local tumors	L.P. fibro. S.	Lung tumors	Per cent lung tumors	Stomach tumors	Per cent stomach tumors
	Br	♀	176	112	63.6	103	58.5	223.1	16	9.1	24	13.6
	Br	♂	147	137	93.2	110	74.8	199.0	13	8.8	73	49.7
Brp	pBr	♀	173	145	83.8	129	74.6	224.0	21	12.1	21	12.1
	pBr	♂	145	118	81.4	97	66.9	205.2	7	4.8	34	23.4
		Total:	641	512	79.9	439	68.5	213.4	57	8.9	152	23.7
	Br	♀	2225	1703	76.5	1557	70.0	237.6	229	10.3	267	12.0
Br	Br	♂	1509	1257	83.3	1071	71.0	211.4	108	7.2	513	34.0
		Total:	3734	2960	79.3	2628	70.4	226.9	337	9.0	780	20.9

* Symbols: Br = dark-eyed, brown, nonagouti; pBr = pink-eyed, brown, nonagouti; S = sarcoma.

in mutants, compared with 79.2 per cent in the parental mice) (Table 1). In each series the local appearing fibrosarcoma is the predominant tumor, 68.5 per cent in mice of the combined Brp subline and 70.4 per cent for mice of the Br subline. This difference is not significant. The incidence and rate of appearance of tumors are presented in Charts 2, 3, 4, and 5. Lung adenomas appeared in 8.8 per cent of mice of the Brp mutant line and in 9.0 per cent of mice of the original Br subline. This difference is also not significant.

The mucosal lesion appeared in 23.7 per cent of the mice of the Brp descent and in 20.9 per cent of mice of the parental Br stock (Table 1 and Chart 6). The gastric lesion appeared more frequently in male mice of all series. This lesion occurred in 12.8 per cent of Brp females and in 36.6 per cent of males of the same subline; the gastric lesion appeared in 12.0 per cent of females of the parental Br stock and in 33.3 per cent of males of the same stock. χ^2 determination on the sex difference for gastric lesions for the Brp descent is 49.6, which gives a P value $<.001$; χ^2 for the sex difference in the original Br subline is 263.3 with a P value $<.001$. Both these differences are significant.

The average latent period for the appearance of fibrosarcomas is approximately the same in mice of all series (Table 1). However, there is a slight difference between the sexes, the tumors appearing somewhat earlier in males of two of the three series (the parental Br and the Br segregates of the mutant Brp descent) than in the three series of females.

DISCUSSION

In the Bl subline, made up of mice descended from a mouse characterized by a dominant mutation at the Black versus Brown locus, there occurred two changes in tumor susceptibilities. One of these changes (susceptibility to induced fibro-

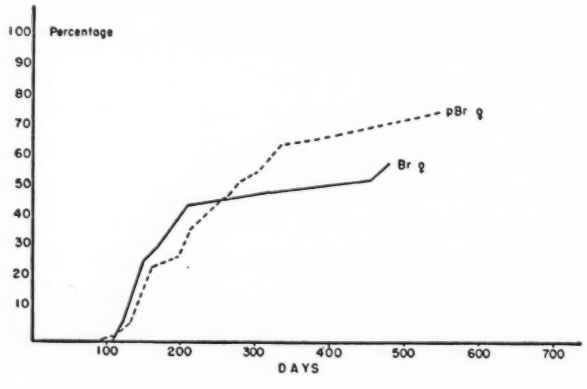

CHART 2.—Rate of appearance of fibrosarcomas at the site of injection of methylcholanthrene in (*a*) Br female (solid line) and (*b*) pBr female of the mutant Brp subline (dash line). Time expressed in days is plotted along the base line; percentage of fibrosarcomas on the vertical line.

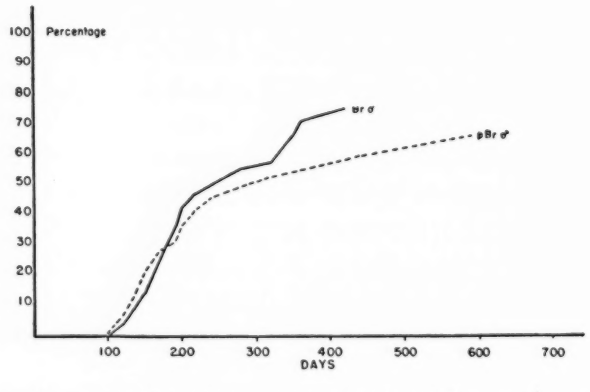

CHART 3.—Rate of appearance of induced fibrosarcomas in (*a*) Br male mice (solid line) and (*b*) pBr male mice (dash line). Time in days is given on the base line; percentage of fibrosarcomas on the vertical line.

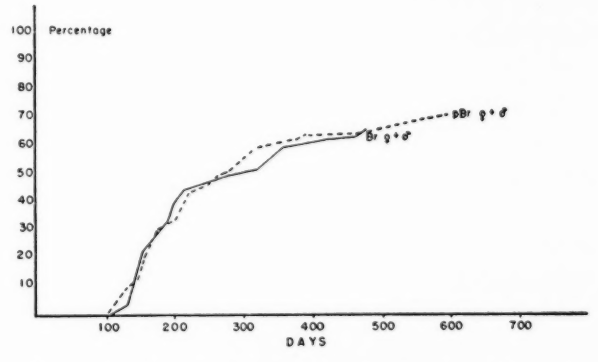

CHART 4.—Data for female and male pBr mice of the mutant Brp subline are added together (dash line) and comparable data for Br female and male mice are also combined (solid line). Time in days is given on base line and percentage incidence of induced fibrosarcomas on the vertical line.

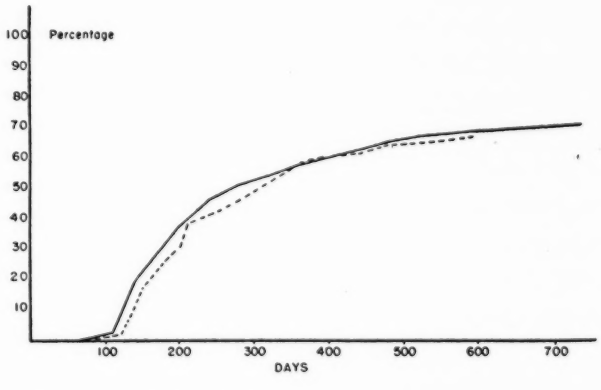

CHART 5.—Induced fibrosarcomas in (*a*) original or ancestral Br subline (solid line) and (*b*) the combined data for all mice of the mutant Brp subline. Time is given in days on the base line; percentage incidence of induced fibrosarcomas on the vertical line.

sarcoma) was in the direction of an increased susceptibility, whereas the other change (a susceptibility to the gastric lesion) was in the opposite direction (6).

In the present experiment, the average latent period for fibrosarcomas for females of the original Br subline was 237.6 days and for males of the same subline, 211.4 days. The average latent period for fibrosarcomas for dark-eyed females of the Brp subline was 223.1 days and for pink-eyed females, 224.0 days; for dark-eyed males, 199.0 days and for pink-eyed males, 205.2 days. Thus, there appears to be no change in susceptibility to fibrosarcomas in the descendants of the pink-eyed mutants and in their litter-mate dark-eyed sibs.

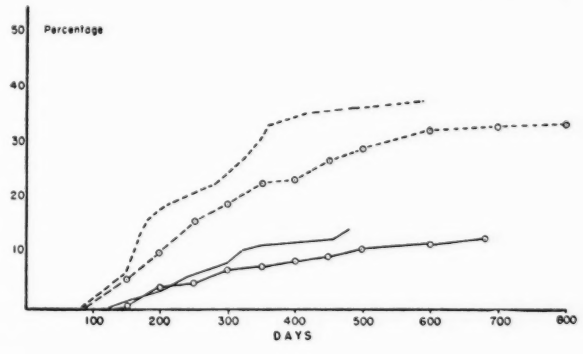

CHART 6.—Data on the rate of appearance of gastric lesions in (*a*) original Br subline females (circle and solid line), (*b*) the original Br subline males (solid line), (*c*) the female mice of the mutant Brp subline (circle and dash line), and (*d*) male mice of the mutant Brp subline (dash line). Time is plotted in days on the base line and percentage incidence of gastric lesions on the vertical line.

The incidence of lung adenomas and the gastric lesion also appear to be unaffected by the mutation at the pink-eye locus. The original sex difference between the incidence of the gastric lesion also appears to be unaffected by the mutation at the "p" locus.

The number of mice developing multiple tumors is the same in the two series. In the Brp descent, 648 lesions (fibrosarcoma, lung adenomas, and gastric lesions) developed in 512 mice, or 1.26 tumors per tumor-bearing mouse, whereas in the parental Br subline 3,745 lesions of the same histological types occurred in 2,860 tumor-bearing mice, or 1.26 tumors per tumor-bearing mouse.

The present data do not give much information on the original nature of an association between pink eye and fibrosarcoma susceptibility. This association appeared to be in the nature of linkage (two genes or entities carried on the same chromosome), but this conclusion may not be the final one. Certainly, it is clear by the present evidence that a mutation may occur at the pink-eye locus without affecting susceptibility to (*a*) fibrosarcoma, (*b*) lung adenoma, and (*c*) a gastric lesion involving the mucosa just anterior to the pylorus.

The present evidence does not indicate whether the new pink-eye mutation should be considered identical to the old pink eye (pp) or to some new allele.

SUMMARY

A mutation at the pink-eye locus occurred in the descendants of dark-eyed mice injected with methylcholanthrene (mutation = P to p). Mice showing the recessive mutation and their dark-eyed sibs continued to develop (*a*) fibrosarcomas at the site of injection of methylcholanthrene, (*b*) lung adenomas, and (*c*) a mucosal lesion just anterior to the pylorus, at the same rate and incidence as mice of their dark-eyed ancestry.

REFERENCES

1. BAGSHAW, M. A. Studies on Gastric Neoplasia in the Mouse. J. Nat. Cancer Inst. (in press).
2. KAPLAN, H. S. Lesions of the Gastric Mucosa in Strong Strain NHO Mice. J. Nat. Cancer Inst., **10**:407–11, 1949.
3. MCPEAK, E., and WARREN, S. The Pathology of Gastric Carcinoma in Mice. J. Nat. Cancer Inst., **7**:309–11, 1947.
4. SMITH, F. W., and STRONG, L. C. Studies on Gastric Neoplasia in Mice. The Histogenesis and Influence of Some Endocrine Factors. J. Nat. Cancer Inst., **10**:423–27, 1949.
5. STRONG, L. C. A Genetic Analysis of the Induction of Tumors by Methylcholanthrene. With a Note on the Origin of the NH Strain of Mice. Am. J. Cancer., **39**:347–49, 1940.
6. ———. Genetic Changes in Gastric Lesion and Fibrosarcoma Susceptibilities. Proc. Soc. Exper. Biol. & Med., **69**:521–24, 1947.
7. ———. A Comparative Study of Susceptibility to Tumors in Mice of Fifteen Inbred Strains (in press).
8. STRONG, L. C.; COLLINS, V. J.; and DURAND, E. A. A Genetic Analysis of the Induction of Tumors by Methylcholanthrene. IV. The Probable Remote Induction of Various Types of Gastric Lesions. Cancer Research, **3**:21–28, 1943.

Carbamates in the Chemotherapy of Leukemia

VIII. Over-all Tracer Studies on Carbonyl-labeled Urethan, Methylene-labeled Urethan, and Methylene-labeled Ethyl Alcohol*

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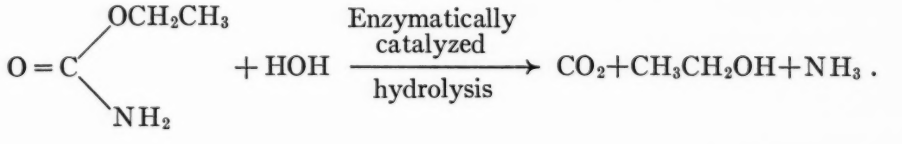
It has been pointed out in previous studies with carbonyl-labeled urethan that on injection this compound begins to break down almost immediately, with about 90 per cent of the carbonyl carbon appearing in the expired carbon dioxide within the first 24 hours. Of the remainder of the carbonyl carbon, 5–10 per cent was found in the urine and about 1 per cent was retained in a fixed form widely distributed in organs, tissues, and body fluids at 24 hours after injection (2). In these earlier studies, it was observed that certain neoplastic mice retained considerably more of the carbonyl carbon atom (in all tissues) at 24 hours. Further investigation with both chemical and tracer technics showed that mice with advanced leukemia and mammary carcinoma catabolize urethan at a slower rate than do normal mice (4). Such findings are in accord with those of Boyland and Rhoden (1).

Earlier results have suggested the general mode of degradation of urethan *in vivo* shown in the reaction below.



The present experiments were planned to learn more of the mode of degradation of urethan in the body and to investigate further the possibility that urethan, or a portion of this molecule, might be localized (in an anatomical sense), thus providing clues to the mechanism of the anti-cancer activity of the drug.

It appeared of special interest to determine the fate of the carbonyl and the methylene carbon atoms of urethan as compared to corresponding atoms from the probable *in vivo* carbon-containing degradation products of this compound, CO_2, and ethyl alcohol.

EXPERIMENTAL

The synthesis of (*a*) carbonyl-labeled and (*b*) methylene-labeled urethan were accomplished through the following reactions:

$$(a)\quad C^{14}O_2 + NH_3 \longrightarrow H_2NC^{14}ONH_2 \xrightarrow{C_2H_5OH} CH_3CH_2OC^{14}ONH_2\,,$$

$$(b)\quad CH_3C^{14}H_2OH + COCl_2 \longrightarrow CH_3C^{14}H_2OCOCl \xrightarrow{NH_3} CH_3C^{14}H_2OCONH_2\,.$$

The details of these procedures will be published elsewhere. The methylene-labeled ethyl alcohol employed was purchased from Tracerlab. The labeled sodium bicarbonate was prepared by the method of Evans and Slotin (3). These carbon 14–labeled compounds were made up for injection at

* This work was supported by the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council, the Institute-Sponsored Research Fund of the Southern Research Institute, and Mr. Ben May, Mobile, Alabama. Certain data included were obtained in a program carried out for the Atomic Energy Commission under a contract with the Office of Naval Research.

Received for publication August 24, 1950.

levels indicated below:

Compound	Structure	Dosage mg/kg	Dosage μc/mouse
Carbonyl-labeled urethan	$O=C^{14}(OC_2H_5)(NH_2)$	1,400	2.0
Labeled sodium bicarbonate	$NaHC^{14}O_3$	100	18.0
Methylene-labeled urethan	$O=C(OC^{14}H_2CH)(NH_2)$	1,400	1.4
Methylene-labeled ethyl alcohol	$HOC^{14}H_2CH_3$	720	1.4

The metabolism chamber used to collect expired CO_2 and excreta from mice injected with the radioactive compounds, the technics for treatment of tissues, and the gas phase counting procedure have been reported previously (5).

Injections were made intraperitoneally, with isotonic saline or distilled water as a carrier, always keeping the volume to 0.2 ml. or less. After injection, the individual mice were immediately placed in the metabolism chamber, and the expired carbon dioxide and excreta were collected at varying times over a 24-hour period. At the termination of an experiment, mice were anesthetized with ethyl ether, exsanguinated, and dissected into tissues of special interest for radioactivity determinations.

Mice employed in these studies were of the Carworth Farms, CFW strain, unless otherwise indicated.

RESULTS

The data obtained with regard to the rate of appearance of carbon 14 in the respiratory carbon dioxide of mice injected with the four compounds are presented in Table 1. These data, as well as results obtained on injection of carbon 14–labeled urea,[1] are plotted for purposes of comparison in Chart 1.

The distribution of carbon 14 in the organs, tissues, and fluids of mice at various periods after injection of carbonyl-labeled urethan and $NaHC^{14}O_3$ have already been reported (1, 6). Similar data on

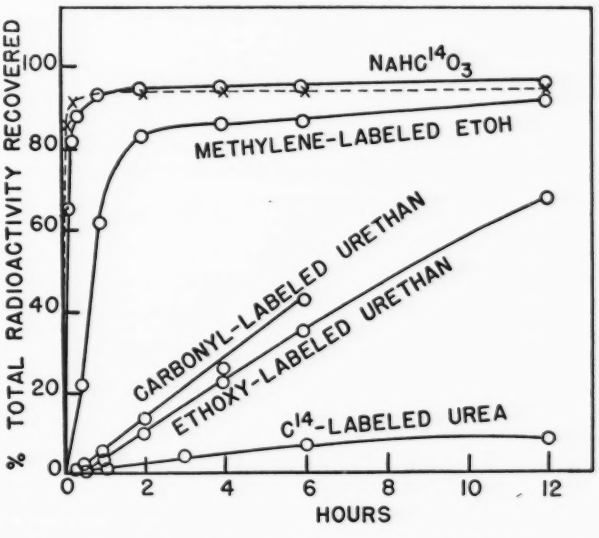


CHART 1.—Rate of expiration of carbon 14 following injection of certain C^{14}-labeled compounds. The broken line for $NaHC^{14}O_3$ is the calculated rate of expiration following continuous injection.

methylene-labeled urethan and methylene-labeled ethyl alcohol, as compared with the above-mentioned compounds, are presented in Table 2.

The total body retention of carbon 14 (in all organs and tissues) at 24 hours after injection of the two labeled urethans and from methylene-labeled ethyl alcohol and $NaHC^{14}O_3$ has been calculated and is summarized in Table 3. Results with regards to the excretion of carbon 14 follow-

[1] Unpublished data.

TABLE 1

RATE OF EXPIRATION OF C^{14} FOLLOWING INJECTION OF CARBONYL-LABELED URETHAN, ETHOXY-LABELED URETHAN, $NaHC^{14}O_3$, AND METHYLENE-LABELED ETHYL ALCOHOL

Period	Cumulative Per Cent of Injected C^{14} Recovered			
	$C_2H_5OOC^{14}NH_2$*	$CH_3C^{14}H_2OOCNH_2$†	$NaHC^{14}O_3$‡	$HOC^{14}H_2CH_3$§
10 min.	0.54		66.0	
20	1.39		82.0	
30	3.2	1.4	88.2	22.6
60	7.0	3.7	92.8	62.2
2 hr.	14.6	10.1	94.1	83.1
3	21.7			
4	27.3	22.3	94.8	86.0
5	33.4			
6	42.4	36.5	95.0	87.4
12		73.0	95.5	
24	98.3	97.4	96.1	92.1

* Average of three experiments.
† Average of three experiments.
‡ Average of two animals in a single experiment.
§ Average of two experiments.

TABLE 2

THE AVERAGE SPECIFIC ACTIVITIES* OF CERTAIN TISSUES 24 HOURS FOLLOWING INJECTION OF C^{14}-LABELED COMPOUNDS

	Compounds and total activities injected				
Tissue	$C_2H_5OOC^{14}NH_2$† (2.0 μc.)	$NaHC^{14}O_3$‡ (18 μc.)	$NaHC^{14}O_3$ (Corrected to 2.0 μc.)§	$CH_3C^{14}H_2OOCNH_2$‖ (1.4 μc.)	$CH_3C^{14}H_2OH$# (1.4 μc.)
Whole blood	0.33	1.09	0.16	0.34	0.36
Blood cells				0.18	0.21
Blood serum				0.44	0.31
Spleen	0.16	0.73	0.11	0.73	0.66
Liver	0.38	1.11	0.16	0.55	0.48
Kidneys	0.16	0.78	0.12	0.52	0.43
Lungs	0.14	0.57	0.08	0.59	0.48
Brain	0.11	0.26	0.04	0.31	0.24
Jejunum	0.37	2.16	0.32	1.29	2.11
Muscle	0.08	0.75	0.11	0.13	0.10
Skin and hair	0.10	0.21	0.03	0.18	0.10
Long bones	0.25	1.01	0.15	0.26	0.25
Marrow				0.27	0.23
Adrenals	0.06			0.16	0.23
Testes	0.18	0.61	0.10	0.27	0.20
Thymus	0.08			0.32	0.29
Lymph nodes	0.11			0.35	0.19

* Specific activities in μc/mole of carbon.

† Three mice used, separate determinations.

‡ Two mice used, pooled tissue samples.

§ Corrected by dividing values obtained on single injection of 18 μc. by 9 and multiplying by 1.36 (calculated correction factor from turnover data).

‖ Five mice used, separate determinations.

Two mice used, separate determinations.

TABLE 3

THE TOTAL BODY RETENTION OF CARBON 14 FOLLOWING INJECTION OF LABELED URETHANS, $NaHC^{14}O_3$, AND $CH_3C^{14}H_2OH$

Compound	Structure	Per cent of total injected C^{14} retained at 24 hours* Individual exp.	Av.
Carbonyl-labeled urethan	$O=C^{14}(OC_2H_5)(NH_2)$	1.4, 1.9, 0.6	1.3
Labeled sodium bicarbonate	$NaHC^{14}O_3$	1.3†	1.3
Ethoxy-labeled urethan	$O=C(OC^{14}H_2CH_3)(NH_2)$	7.87,‡ 6.71, 7.41	7.3
Methylene-labeled ethyl alcohol	$HOC^{14}H_2CH_3$	7.6, 4.3, 5.21, 7.68	6.2

* Arrived at by determination of the sum of the total radioactivity in various organs and tissues and dividing by the dose (in μc.) injected.

† Assays carried out on pooled samples from two mice.

‡ The 7.87 value was obtained on pooled tissues of three mice.

Note: These retention values were based on actual tissue weights, tissue carbon determinations, and tissue specific activities.

ing injection of the labeled compounds are presented in Table 4.

In an additional study of the rate of catabolism of urethan by normal and neoplastic mice, a comparison of the retention of radioactive carbon from methylene-labeled urethan by normal mice and an animal with spontaneous mammary carcinoma has been carried out (Table 5). In this same table a comparison of the carbon dioxide fixation of normal and a leukemic mouse is presented.

DISCUSSION

This rather extensive study of the fate of two important atoms of the urethan molecule has been conducted as one phase of a program designed to shed light on the mechanism of action of this compound. Since urethan appears to undergo hydrolysis in the body, releasing carbon dioxide, ethyl alcohol, and ammonia, data on the fixation and distribution of the corresponding atoms in these hydrolysis products are necessary before conclusions may be drawn regarding the significance of tracer studies with this compound.

TABLE 4

THE EXCRETION OF C^{14} FOR THE FIRST 24 HOURS AFTER INJECTION OF VARIOUS LABELED COMPOUNDS

COMPOUND	AVERAGE PER CENT OF TOTAL DOSE RECOVERED IN: Urine	Feces
Carbonyl-labeled urethan	7.82	<1.0
$NaHC^{14}O_3$	1.35	<1.0
Ethoxy-labeled urethan	3.31	0.02
$CH_3C^{14}H_2OH$	3.70	0.24
C^{14} urea	80.0	<1.0

The rate of catabolism of urethan as determined by chemical analyses of blood from mice injected intraperitoneally has been reported (4). It is apparent from these data and from results obtained on the rate of appearance of carbon 14 in the respiratory carbon dioxide of mice injected with the labeled urethans, $NaHC^{14}O_3$ and C^{14}-labeled ethyl alcohol (Chart 1) that, in order to compare the results on carbon 14 retention obtained with these four compounds, the kinetics of carbon turnover must be considered. In the case of $NaHC^{14}O_3$, more than 90 per cent of the radioactive carbon is lost in the respiratory carbon dioxide within an hour after injection. It is assumed that $C^{14}O_2$ from the hydrolysis of carbonyl-labeled urethan is released continuously over a number of hours at a rate which decreases with time. The rate of hydrolysis is not known. However, it has been reported earlier that the hydrolysis of urethan is essentially complete within 18 hours. Therefore, since the rate of hydrolysis decreases with time, it is safe to assume that the retention of C^{14} from the hydrolysis will be intermediate between that for a single dose of bicarbonate and that for continuous injection of bicarbonate over a period of 18 hours. On the basis of a highly simplified mechanism, the retention resulting from a continuous 18-hour injection has been estimated.

TABLE 5

THE SPECIFIC ACTIVITY* OF CERTAIN MOUSE TISSUES FROM NORMAL AND NEOPLASTIC MICE AT 24 HOURS AFTER INJECTION OF METHYLENE-LABELED URETHAN AND $NaHC^{14}O_3$

	$NH_2COOC^{14}H_2CH_3$ (1.4 μc.)		$NaHC^{14}O_3$ (18 μc.)	
TISSUE	Normal	Tumor-bearing†	Normal	Leukemic‡
Blood	0.34	3.02	1.09	0.37
Blood serum	0.44	3.41		
Blood cells	0.18	1.43		
Spleen	0.73	2.21	0.73	1.47
Liver	0.55	1.30	1.11	1.24
Kidneys	0.52	1.37	0.78	0.58
Lungs	0.59	1.52	0.57	0.66
Brain	0.31	0.76	0.26	0.50
Jejunum	1.29	1.79	2.16	0.99
Muscle	0.13	1.10	0.75	0.35
Skin and hair	0.18	0.81	0.21	0.34
Long bones	0.26	0.74	1.01	0.80
Bone marrow	0.27	0.44		
Mammary tumor		1.74		
Control mammary tissue		0.35		
Adrenals	0.16	0.27		
Thymus	0.32	0.82		
Lymph nodes	0.35	0.10		
Per cent of total C^{14} retained in all tissues:	7.3	36.0	1.37	0.87

* Specific activity in μc/mole of carbon.

† CFW strain mouse with spontaneous mammary carcinoma.

‡ Advanced spontaneous leukemia in an Akm mouse.

For this estimate, it was assumed that C^{14} from bicarbonate can be involved in the following three reactions:

$$\text{carbon dioxide } (C) \xleftarrow{k_1} \text{bicarbonate } (B) \underset{k}{\overset{k_2}{\rightleftharpoons}} \text{tissue } (T).$$

Rate constants, k_1, k_2, and k_3, were evaluated with data obtained from mice receiving only a single instantaneous injection. Then these constants were used to calculate the retention from an 18-hour injection.

For a single injection of bicarbonate, the differential equations describing the situation are:

$$\frac{dC}{dt} = k_1 B\,,$$

$$\frac{dB}{dt} = -k_1 B - k_2 B + k_3 T\,, \qquad (1)$$

$$\frac{dB}{dt} + \frac{dC}{dt} + \frac{dT}{dt} = 0\,. \qquad (2)$$

These can be combined to yield:

$$\frac{d^2B}{dt^2} + (k_1 + k_2 + k_3)\frac{dB}{dt} + k_1 k_3 B = 0\,,$$

for which the general solution is:

$$\left.\begin{aligned} B = {} & A_1 \exp\left(-b - \sqrt{b^2-\omega^2}\right) t \\ & + A_2 \exp\left(-b + \sqrt{b^2-\omega^2}\right) t\,, \end{aligned}\right\} \qquad (3)$$

with

$$2b = k_1 + k_2 + k_3$$

and

$$\omega^2 = k_1 k_3\,.$$

With this solution for B, T can be obtained from equation (1) by differentiation to yield:

$$\left.\begin{aligned} T = {} & \frac{A_1}{k_3}\left(k_1 + k_2 - b - \sqrt{b^2-\omega^2}\right) \\ & \exp\left(-b - \sqrt{b^2-\omega^2}\right) t + \frac{A_2}{k_3}\left(k_1 + k_2\right. \\ & \left. - b + \sqrt{b^2-\omega^2}\right) \exp\left(-b + \sqrt{b^2-\omega^2}\right) t\,. \end{aligned}\right\} \qquad (4)$$

If the concentration units for B, C, and T are per cent of total dose per mouse, then:

$$C = 100 - (B + T)$$

or

$$\left.\begin{aligned} C = {} & 100 - \frac{A_1}{k_3}\left[k_1 + k_2 + k_3 - b - \sqrt{b^2-\omega^2}\right] \\ & \times \exp\left(-b - \sqrt{b^2-\omega^2}\right) t - \frac{A_2}{k_3}\left[k_1 + k_2 + k_3\right. \\ & \left. - b + \sqrt{b^2-\omega^2}\right] \exp\left(-b + \sqrt{b^2-\omega^2}\right) t\,. \end{aligned}\right\} \qquad (5)$$

It is more convenient, in evaluating the constants, to leave equation (5) expressed in terms of b and ω as well as the rate constants, rather than to convert b and ω into the equivalent expressions in terms of the rate constants.

For the evaluation of the constants, the rate of increase of C for the first few minutes after injection is the most sensitive measure of the first, larger exponent, and the rate of increase of C several hours later is the best measure of the second, smaller exponent. The coefficient of the second exponential term, which comes directly from the expression for $B + T$, is best evaluated by the total retained radioactive carbon when the animal is sacrificed at the end of the experiment. The sum of the two coefficients in equation (5) must equal 100, and so must the sum of A_1 and A_2. These five equations, with the two defining b and ω, are sufficient to evaluate the two A's, three k's, b, and ω.

The constants will vary, of course, from animal to animal. However, composite values taken from data for several mice are as follows: $A_1 = 99.9876$ per cent of dose; $A_2 = 0.0124$ per cent of dose; $k_1 = 6.764$ hr.$^{-1}$; $k_2 = 0.445$ hr.$^{-1}$; and $k_3 = 0.0157$ hr.$^{-1}$.[2] The dashed curve for $NaHCO_3$ in Chart 1 is calculated from these constants. The measured points on the $NaHCO_3$ curve came from two mice. It is evident from the curve, and especially from a comparison with the retention data for longer periods, that the assumed mechanism is deficient in not providing at least one more form of retained C^{14}; with an additional pair of rate constants for that form, the curve could be fitted much more closely. However, since the analytical data do not distinguish between types of retained C^{14}, introduction of another type of tissue into the mechanism would not be susceptible to test by the existing data.

In spite of the limitations of the assumed mechanism, the agreement over periods of the first few days is good enough to give some support to calculations of retention from continuous injections. In this case, during the course of the injection, equations (1) and (2) become, respectively:

$$\frac{dB}{dt} = -k_1 B - k_2 B + k_3 T + k_4$$

and

$$\frac{dB}{dt} + \frac{dC}{dt} + \frac{dT}{dt} = k_4\,.$$

The general solution for B is identical with equation (3), except that the term k_4/k_1 is added on the right. Correspondingly, $(k_2 k_4)/(k_1 k_3)$ must be added on the right of equation (4). After the injection is completed, k_4 becomes zero and equations (3), (4), and (5) apply again.

For calculations involving continuous injection,

[2] It is not intended to imply that the constants have been evaluated with the precision quoted. These are simply the exact values of the constants used to calculate the dashed curve in Chart 1.

the coefficients of the exponential terms must be evaluated again, using the facts that B, C, and T are all zero at the start. The original rate constants are unchanged, and k_4 is determined by the rate of injection.

The result of the calculation for an 18-hour injection, using the rate constants quoted above, is that 5.5 per cent would be retained at 24 hours. This is to be compared with a value of 4.0 per cent for an instantaneous injection. Therefore, within the limitations of the mechanism assumed, it is clear that the amount of C^{14} retained after 24 hours is relatively independent of whether it comes directly from $NaHC^{14}O_3$ or by hydrolysis of carbonyl-labeled urethan, since the retention after hydrolysis will be intermediate between that for instantaneous and continuous injections.

The data presented in Chart 1 and in Tables 1–4 are compatible with the postulate that the principal mode of degradation of urethan in the body is a simple hydrolysis. It would appear that the difference in the rate of appearance of C^{14} in respiratory carbon dioxide of mice injected with carbonyl-labeled and methylene-labeled urethan might be accounted for by the time required to oxidize the methylene-labeled ethyl alcohol (produced as a result of hydrolysis of ethoxy-labeled urethan) to carbon dioxide.

It can be seen from the results summarized in Table 3 that the total body retention of the carbonyl carbon from urethan might be accounted for by carbon dioxide fixation. More of the methylene carbon from ethyl alcohol was retained by mice than could be expected from fixation of CO_2, the final oxidation product of this alcohol. The similarity in over-all body retention of the methylene carbon from methylene-labeled urethan and methylene-labeled ethyl alcohol suggests that the fixation of the labeled atom of the former compound might be accounted for by fixation of the isotope from the urethan hydrolysis product, methylene-labeled ethyl alcohol. The consistent retention of a greater percentage of C^{14} from methylene-labeled urethan and methylene-labeled ethyl alcohol indicates the entrance of a small amount of the methylene carbon of ethyl alcohol into the metabolic pathways prior to oxidation of this atom to carbon dioxide. *In vivo* oxidation of some of the alcohol to acetate which enters normal metabolic pathways could account for the higher per cent retention of C^{14} from labeled ethyl alcohol than was observed on injection of $NaHC^{14}O_3$.

The distribution of the C^{14} from the four compounds in the tissues of mice at 24 hours adds further strength to the suggested mode of urethan degradation. It can be seen in Table 2 that there is a certain correlation between the distribution ratio of C^{14} from carbonyl-labeled urethan and $NaHC^{14}O_3$. The distribution of C^{14} among the tissues and organs from mice receiving methylene-labeled urethan and labeled ethyl alcohol likewise follows the same general pattern.

Our further investigation of the rate of catabolism of urethan in normal and neoplastic mice has shown that the methylene carbon of the ethoxy group in urethan was excreted much less rapidly in a mouse with mammary carcinoma than in the case of normal mice (Table 5). This confirms earlier observations of a slower rate of hydrolysis of urethan in neoplastic than in normal mice (2, 4). In a single experiment also reported in Table 5 no profound difference in carbon dioxide fixation was observed in normal and leukemic mice.

SUMMARY

Over-all tracer studies have been carried out on carbonyl- and methylene-labeled urethan, $NaHC^{14}O_3$, and methylene-labeled ethyl alcohol.

The rate of expiration of C^{14} following injection of these compounds, the percentage of the total activity retained by mice at 24 hours, and the general distribution of radioactivity suggest that urethan is eventually hydrolyzed *in vivo* to carbon dioxide, ethyl alcohol, and ammonia.

A mouse with advanced spontaneous mammary carcinoma has been observed to retain 36 per cent of the C^{14} from methylene-labeled urethan at 24 hours, while normal controls retained but about 7.3 per cent of the same atom from this radioactive compound.

REFERENCES

1. BOYLAND, E., and RHODEN, E. The Distribution of Urethane in Animal Tissue, as Determined by a Microdiffusion Method, and the Effect of Urethane Treatment on Enzymes. Biochem. J., **44**:528–31, 1949.
2. BRYAN, C. E.; SKIPPER, H. E.; and WHITE, L., JR. Carbamates in the Chemotherapy of Leucemia. IV. The Distribution of Radioactivity in Tissues of Mice Following Injection of Carbonyl-labeled Urethane. J. Biol. Chem., **177**:941–50, 1949.
3. EVANS, E. A., JR., and SLOTIN, L. Carbon Dioxide Utilization by Pigeon Liver. J. Biol. Chem., **141**:439–50, 1941.
4. MITCHELL, J. H., JR.; HUTCHISON, O. S.; SKIPPER, H. E.; and BRYAN, C. E. Carbamates in the Chemotherapy of Leucemia. VII. The Rate of Catabolism of Urethane in Normal and Neoplastic Mice. J. Biol. Chem., **180**:675–80, 1949.
5. SKIPPER, H. E.; BRYAN, C. E.; WHITE, L., JR.; and HUTCHISON, O. S. Techniques for *in vivo* Tracer Studies with Radioactive Carbon. J. Biol. Chem., **173**:371–81, 1948.
6. SKIPPER, H. E.; WHITE, L., JR.; and BRYAN, C. E. Studies on the Hazard Involved in Use of C^{14}. I. Retention of Carbon from Labeled Sodium Bicarbonate. J. Biol. Chem., **180**: 1187–95, 1949.

The Effect of Estrogen on the Serum Glucuronidase Activity of Patients with Breast Cancer*

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A relationship between estrogen and the β-glucuronidase activity of the uterus was first suggested by Fishman (2, 5). He reported that a decreased glucuronidase activity of the uterus in the mouse followed ovariectomy and that this activity was markedly increased by subsequent treatment of the animals with estrogens. A similar estrogen-β-glucuronidase relationship for other sexual tissues has been suggested by the following observations: (*a*) Fishman and Anlyan (4) reported a greatly increased glucuronidase activity in the human mammary gland during lactation and in breast carcinomas; (*b*) Odell, Burt, and Bethea (11) observed an increase in the glucuronidase activity in the cervix of women during pregnancy and in both the cervix and the vaginal fluid of untreated lower genital tract carcinoma. Kerr, Levvy, and Campbell (8) have suggested that the increased glucuronidase activity effected by estrogens and present in malignancies is a generalized phenomenon, in that they have observed an increased β-glucuronidase in a wide variety of tissues in which mitosis has been stimulated. Fishman and Anlyan (4) also observed an increased glucuronidase activity in a number of cancers involving nonsexual tissues; they point out, however, that in none of these cases is the increase in glucuronidase activity as great as that found in the mammary carcinoma.

There is no direct evidence that an increase in the β-glucuronidase activity of the blood occurs when a high level of estrogen is present in the body or in the presence of malignancies. Two indirect indications of such a relationship have, however, been observed: (*a*) Fishman (3) and McDonald and Odell (9) all reported a rise in the blood levels of β-glucuronidase during pregnancy and obtained curves which paralleled the increased estrogen production that has long been known to occur in pregnancy; and (*b*) Fishman, Odell, Gill, and Christensen (6) have recently reported that the daily administration of 5–25 mg. of stilbestrol to *post partum* women caused a significant reduction in the rate at which the blood glucuronidase level fell from the high *ante partum* levels.

The data presented in this paper offer the first direct evidence that the administration of estrogens can cause an increase in the glucuronidase activity of blood serum. These data were obtained during the course of studies on the metabolic effects of estrogen therapy on patients with advanced breast carcinoma.

METHODS

This report includes all the blood glucuronidase data of the mammary cancer patients thus far studied by us. These patients were selected in accord with the suggestions of the Subcommittee on Steroids and Cancer of the Therapeutic Trials Committee of the A.M.A. (1947). The patients selected thus all had advanced breast cancer and were at least 5 years post-menopausal.

During their course of therapy, eight of the patients were treated daily with 3 mg. ethinyl estradiol administered orally, and six of the patients were similarly treated with 15 mg. of diethylstilbestrol.[1] Control blood samples were drawn prior to the initiation of therapy and at intervals thereafter. In addition, a number of assays were carried out on blood samples from individuals being examined in the University of Minnesota Cancer Detection Center. These latter patients were of about the same age as those receiving the estrogen therapy and offered a means of comparing the serum glucuronidase levels of healthy individuals with those of patients with breast cancer.

* This work was aided by grants from the American Cancer Society upon recommendation of the Committee on Growth, The Minnesota Cancer Society, and the University of Minnesota Graduate School.

Received for publication August 28, 1950.

[1] The hormone preparations used in this study were supplied through the Therapeutic Trials Committee of the American Medical Association by the Abbott Research Laboratories, Schering Corporation, and Winthrop-Sterns, Inc.

Serum glucuronidase activity was determined by the method of Fishman, Springer, and Brunetti (7).

RESULTS

Pre-therapy serum glucuronidase levels.—The blood serum levels of β-glucuronidase activity of patients with mammary cancer and of the noncancerous controls are shown in Table 1. It is seen that no significant difference exists in the values for the cancerous and noncancerous females. The data do, however, indicate significantly higher serum glucuronidase levels for men than for women in this age group.

TABLE 1

THE β-GLUCURONIDASE ACTIVITY OF SERA OF CONTROL AND MAMMARY-CANCEROUS PATIENTS

	No. of patients	Age of patients		Glucuronidase activity of blood sera (units per cent)		
		Range	Average	Range	Average	Standard error
Untreated patients:						
Male controls	16	47–63	54	640–1,360	926	44
Female controls	33	43–84	55	300–1,100	670	41
Patients with mammary cancer not treated with estrogen	12	42–70	57	350–1,520	770	97
Estrogen-treated patients:						
Average pre-therapy levels for patients with mammary cancer	14	54–73	64	300–1,160	770	68
Maximum values attained on stilbestrol therapy	6	59–73	67	1,260–1,900	1,670	63
Maximum values attained on ethinyl estradiol therapy	8	54–70	61	1,240–3,000	1,750	204

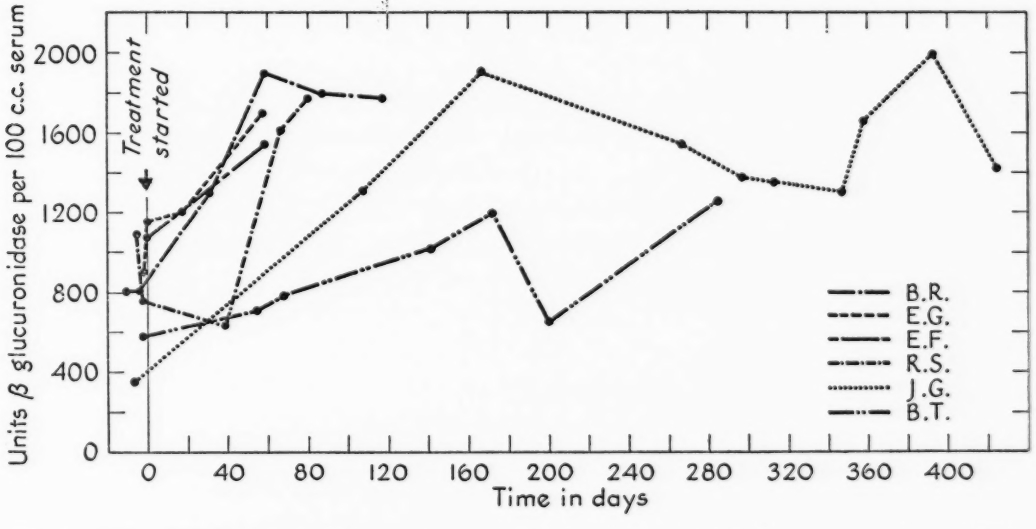

FIG. 1.—The effect of stilbestrol therapy on serum β-glucuronidase levels of mammary cancer patients

Serum glucuronidase levels during estrogen therapy.—In Figures 1 and 2 are shown the serum glucuronidase levels obtained during the course of therapy with diethylstilbestrol and with ethinyl estradiol, respectively. It can be seen that a significant increase in serum β-glucuronidase activity occurred in thirteen of the fourteen mammary cancer patients thus treated with estrogen. All the estrogen-treated patients attained serum β-glucuronidase levels of at least 1,200 units per cent, a value exceeded by only 4 (2 untreated mammary cancer patients and 2 normal males) of the 75 patients in the over-50 age group on whom we have conducted serum glucuronidase assays in our laboratory up to the present time. The average maximum serum glucuronidase attained is about 1,700 units per cent, which level represents an increase of more than 100 per cent over pre-therapy levels. One patient on stilbestrol therapy (Patient J. G., Fig. 1) for over a year still shows an average serum glucuronidase value of 1,600 units per cent (1,300–2,000), a value which represents an increase of 300+ per cent over her pre-therapy determination. There appears to be no obvious relationship between the progress of the malignancy and the

serum glucuronidase levels during estrogen therapy.

DISCUSSION

While there seems to be a somewhat greater serum β-glucuronidase activity in the patients with the mammary cancers than in the normal controls, this difference is not statistically significant for the patients thus far studied. This observation is in agreement with the report of Fishman and Anlyan (4) that there was no consistent increase in the serum glucuronidase levels of their patients with mammary carcinomas.

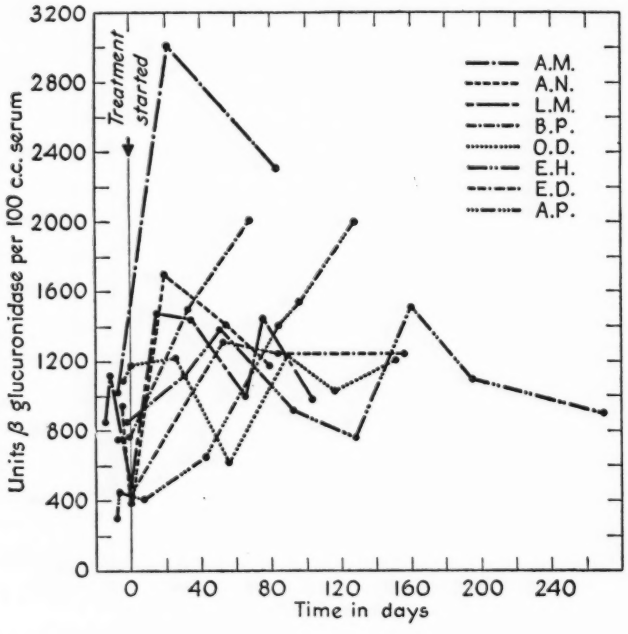

FIG. 2.—The effect of ethinyl estradiol therapy on serum β-glucuronidase levels of mammary cancer patients.

It is obvious from the data presented in this paper that estrogen therapy administered to patients with mammary cancer causes an increase in the level of serum β-glucuronidase activity. This increase is usually quite marked within 3–4 weeks and is apparently maintained as long as the estrogen therapy is continued. No significant difference was observed in the group treated with stilbestrol, as compared to that receiving ethinyl estradiol as the estrogenic substance. It is hoped to determine the importance of the cancerous mammary gland in this response to estrogen by studies on the serum glucuronidase activity of other patients being treated with estrogens.

While we have had occasion to make observations on only two patients with mammary cancer on testosterone therapy, in neither of these cases was there any significant effect on the serum glucuronidase activity. One of these cases was treated with 100 mg. testosterone 3 times a week for 14 months, at the end of which time her serum glucuronidase was only 830 units per cent, whereas after 2 months on stilbestrol therapy her serum glucuronidase had attained a level of 1,900 units per cent (Patient B. R., Fig. 1).

The apparently greater serum glucuronidase activity in men than in women in the post-age-50 group is difficult to explain by either the tissue growth (8) or the metabolic conjugation (3) hypotheses elaborated for tissue glucuronidase by these authors.

On the basis of data presented in this paper it would seem desirable to determine the effect of age and sex on serum glucuronidase activity levels.

SUMMARY

1. No significant difference in the serum β-glucuronidase levels of normal women and women with mammary cancers was observed.

2. Male control patients have a significantly higher serum glucuronidase activity level than do women in the post-age-50 group.

3. Estrogen therapy causes a marked rise in the β-glucuronidase activity in the sera of patients being treated for mammary cancer. The average maximum values for all patients studied was more than twice as high as the pre-therapy levels.

4. No difference could be observed in the serum β-glucuronidase response for the diethylstilbestrol and the ethinyl estradiol-treated groups.

ACKNOWLEDGMENT

We wish to thank Shirley Tennyson and Ruth Harris for technical assistance in carrying out the assays reported in this paper.

REFERENCES

1. A.M.A. COUNCIL IN PHARM. AND CHEM. Estrogens and Androgens in Mammary Cancer. J.A.M.A., **135**:987–89, 1947.
2. FISHMAN, W. H. β-Glucuronidase: Its Relation to the Action of the Estrogenic Hormones. J. Biol. Chem., **169**: 7–15, 1947.
3. ———. β-Glucuronidase Activity of the Blood and Tissues of Obstetrical and Surgical Patients. Science, **105**:646–47, 1947.
4. FISHMAN, W. H., and ANLYAN, A. J. β-Glucuronidase Activity in Human Tissues. Some Correlations with Processes of Malignant Growth and with the Physiology of Reproduction. Cancer Research, **7**:808–17, 1947.
5. FISHMAN, W. H., and FISHMAN, L. W. The Elevation of Uterine β-Glucuronidase Activity by Estrogenic Hormones. J. Biol. Chem., **152**:487–88, 1944.
6. FISHMAN, W. H.; ODELL, L. D.; GILL, J. E.; and CHRISTENSEN, R. A. The Influence of Stilbestrol on Serum β-Glucuronidase in Women following Parturition. Am. J. Obst. & Gynec., **59**:414–18, 1950.

7. FISHMAN, W. H.; SPRINGER, B.; and BRUNETTI, R. Application of an Improved Glucuronidase Assay Method to the Study of Human Blood β-Glucuronidase. J. Biol. Chem., **173**:449–56, 1948.
8. KERR, L. M. H.; LEVVY, G. A.; and CAMPBELL, J. G. β-Glucuronidase and Tissue Proliferation. Nature, **160**:572, 1947.
9. MCDONALD, D. F., and ODELL, L. D. Serum Glucuronidase Activity during Normal and Toxemic Pregnancy. J. Clin. Endocrinol., **8**:535–42, 1947.
10. ODELL, L. D., and BURT, J. C. β-Glucuronidase Activity in Human Female Genital Cancer. Cancer Research, **9**: 362–65, 1949.
11. ODELL, L. D.; BURT, J. C.; and BETHEA, R. β-Glucuronidase Activity. Science, **109**:564–65, 1949.

Summation and Inhibition Effects of Weak and Strong Carcinogenic Hydrocarbons: 1:2-Benzanthracene, Chrysene, 1:2:5:6-Dibenzanthracene, and 20-Methylcholanthrene*

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INTRODUCTION

The summation or additive effects of carcinogenic agents have received much attention in efforts made to understand the action of carcinogens and the events that occur in carcinogenesis (5, 21). Chemical carcinogens, tumor-producing viruses, radiant energy, and trauma have been tried in various combinations (46). Some agents showed summation, others did not, and a third group showed inhibition. The present report is concerned with combinations of chemical agents only.

The earliest work with summation of chemical carcinogens was done with tars (5) or other mixtures. After the carcinogenic hydrocarbons were discovered in 1931, the first summation experiment with these compounds was made by Hieger (24). He found that one strong carcinogen (1:2:5:6-dibenzanthracene) could complete the carcinogenic action on skin begun by another (3:4-benzpyrene). Lavik *et al.* reported that methylcholanthrene, benzpyrene, and dibenzanthracene administered in subcarcinogenic doses could elicit tumors following methylcholanthrene preparation (35). Rusch *et al.* observed that combinations of chemicals were additive in carcinogenesis on mouse skin but that chemicals and ultraviolet light, Shope virus and ultraviolet light, and Shope virus and x-ray irradiation did not summate (46). No addition was observed by Jaffé when two carcinogens of a different chemical nature were used (27, 28). Thus, neither urethan nor azo dyes were additive to methylcholanthrene. Stasney *et al.* observed augmentation of liver tumors in rats fed 2-acetaminofluorene if estradiol or pregnant mare serum gonadotrophin were also administered (49). It is assumed that in those experiments where summation did not occur its absence was due to failure of the agents to act on the same intracellular receptors.

Inhibition of one chemical carcinogen by another chemical has also been demonstrated a number of times. Experiments in which the exact chemical nature of either agent was unknown are omitted from the present discussion. For experiments where both agents were pure chemicals—using 20-methylcholanthrene, 3:4-benzpyrene and 1:2:5:6-dibenzanthracene as inductors—the inhibitors can be divided into four classes; namely, hydrochloric acid–liberating compounds (6, 15), aliphatic aldehydes (13, 16, 44, 45), unsaturated dibasic acids (17), and aromatic compounds (18, 19, 34, 53). All four classes seem to interfere with the sulfur metabolism of the cell. Some of the inhibiting compounds of the aromatic series are closely related to actual carcinogens, suggesting that the mode of action is a competition for available SH groups.

The present report concerns summation and inhibition effects by new chemical combinations. The experiments were planned to cover the interactions of extremes in potency; two weak, two strong, and a weak plus a strong chemical carcinogen were used. The strong carcinogens (1:2:5:6-dibenzanthracene and 20-methylcholanthrene) were chosen because of the large amount of careful work done by others on titrating the 50 per cent tumor dose (TD_{50}). The weak carcinogens (chrysene and 1:2-benzanthracene) were selected because they represent two basic ring structures from which nearly all carcinogenic hydrocarbons are derived and because they have often been reported to be carcinogenically inert.

Thirteen tests of the carcinogenicity of 1:2-benzanthracene have been reported. Most workers have found it to be inactive (1, 8, 10, 24, 36, 39, 40, 48, 55). However, Kennaway (30) produced one transitory papilloma in fifty mice in an unspecified time. Cook, in 1933 (14), obtained one epithelioma in eighty mice, also in an unspecified

* This investigation was aided by a research grant from the American Cancer Society, on recommendation by the Committee on Growth of the National Research Council.

Received for publication August 30, 1950.

time. Barry *et al.* (4) found one epithelioma among thirty mice painted for periods up to 584 days. White and Eschenbrenner (54) fed this chemical to six rats for 14 months and observed two hepatomas. From the collected experiments, it can be concluded that weak cancer-producing activity has been shown for this compound.

Much to our surprise, 1:2-benzanthracene proved to be a fairly potent carcinogen. The specimen used was a commercial product. Spectrophotometric examination of fractions obtained by chromatography showed the ultraviolet absorption spectrum of 1:2-benzanthracene, and not that of any other known carcinogen. The discovery that 1:2-benzanthracene was definitely carcinogenic appeared of such great importance for our work, and in explaining hitherto contradictory results observed by others in collateral lines of research (see "Discussion"), that the experiments were immediately repeated and extended. The final results of these new tests are not yet available, but they already confirm on a large scale the carcinogenicity of 1:2-benzanthracene; they will be reported on completion. For present purposes it should be emphasized that our confidence in the carcinogenicity of 1:2-benzanthracene is based on far more extensive data than are presented in this paper.

Chrysene has been tested for carcinogenicity many times. Most investigators have reported it to be inactive (10, 23, 31–33, 37–39, 40–42, 47, 48). A few, however, found it to possess mild activity. Twort and Fulton, in 1930 (51), induced three tumors in 70–85 weeks in an unstated number of surviving mice by skin-painting chrysene in liquid paraffin or in oleic acid. Cook (14) found three sarcomas in ten rats injected with chrysene in a fatty medium. Bottomley and Twort (9) skin-painted mice with chrysene in various solvents for periods of 50–78 weeks and obtained thirteen tumors, of which nine were found in tests with oleic acid as the solvent. Barry and Cook (3) tested chrysene by subcutaneous injection and found four malignant tumors in ten rats within 20 months. That sample of chrysene may have been impure. Barry *et al.* (4) painted the skins of 300 mice for periods lasting from 440 to 797 days and observed one epithelioma and three papillomas. Bachmann *et al.* (2) saw one papilloma on a mouse on the 853d day. Twort and Twort (52) painted mice with chrysene in oleic acid or chloroform and obtained tumors with the former. The substance was regarded as one-tenth as potent as 1:2:5:6-dibenzanthracene in oleic acid.

The strong carcinogenic potencies of 20-methylcholanthrene and of 1:2:5:6-dibenzanthracene are well known. An attempt was made to find and use the amount that would produce tumors in 50 per cent of the animals, so that either summation or inhibition effects could be recognized (11, 12).

METHODS

Fifty C57 black mice, the second and third generation descendants of breeding stock obtained from the Roscoe B. Jackson Memorial Laboratory, 3–4 months old and about equally divided as to sex, were used in each experiment. They were fed laboratory chow pellets and water and were housed with ten to fifteen in a cage. Weights were taken at monthly intervals. The observed differences in tumor yield are not explained by inequalities in animal weight or by sex, so that these two factors are not mentioned again.

The mice received subcutaneous injections in the interscapular region with the designated amount of chemical in 0.5 cc. tricaprylin. The mice were observed weekly for a month, to record the amount of loss by early ulceration; thereafter, they were observed monthly until tumors appeared, after which period weekly observations were resumed until the experiments were terminated in the 22d month. About 5 per cent of the mice in each experiment, including the tricaprylin controls, lost their chemical soon after injection, as indicated by sloughs and by failure to show a residual deposit at necropsy. The loss appeared to be about equal in all groups. All mice were examined at autopsy, and all suspicious lesions and tumors were examined on microscopic sections. Only sarcomas at the site of injection were counted as induced tumors. In many instances, the residual oil and chemical mass was saved at necropsy for spectrophotometric analysis, and many injection masses were saved for histological study of the cellular reaction. Spectrophotometric examination of residues of injected materials was made in fifteen mice. In fourteen, the presence of the expected chemical was demonstrated; in one mouse there was a 1-mm. deposit of clear oil, but no chemical could be detected. Most of these analyses were done at or near the end of the experiments.

Three of the chemicals (1:2-benzanthracene, 20-methylcholanthrene, and 1:2:5:6-dibenzanthracene) were commercial products obtained from the Eastman Kodak Company. Chrysene was obtained from the Reilly Tar and Chemical Corporation. The purity of the 1:2-benzanthracene has already been described. The other three chemicals were not purified, but previous studies by spectrophotometry following chromatography had shown absence of absorption spectra of other carcinogenic hydrocarbons.

Because, according to the reports in the literature, chrysene and 1:2-benzanthracene were questionably carcinogenic, the large dose of 5.0 mg. per mouse was selected to assure a saturation or excessive dose of the compounds. In the case of the strong carcinogens, where addition effects, if they existed, were sought, the median tumor dose (TD_{50}) was used. The results of the tests, therefore, are not directly comparable, because the quantity of the injected chemicals varied from TD_{50} to TD_{100}. For the same reason the carcinogenic index of Iball (25) and the carcinogenic grade of Berenblum (7) are not directly determinable, from the data, for all chemicals.

rent infections were not equal in all experiments. This table shows the number of survivors and the deaths from tumors for each month. The difference between these figures represents death from other causes. Survival was poorest in the 1:2-benzanthracene experiment.

Controls.—No sarcomas were found in the uninjected controls. In our experience, spontaneous fibrosarcomas occur very rarely in the C57 black strain—and then only in old mice and usually in the extremities rather than on the trunk, where tumors are induced.

Three sarcomas were found in the tricaprylin controls, constituting an incidence of about 1 per

TABLE 1

CARCINOGENICITY AND COMBINATION EFFECTS OF HYDROCARBONS

Compound tested	Amt. of chemical	No. of mice	Effective total mice*	Effective total mice†	No. of induced sarcomas	Tumor yield‡ (per cent)	Tumor yield§ (per cent)	Minimum induction time (days)	Av. induction time (days)
Chrysene	5.0 mg.	50	39	24	4	10.3	16.6	349	401
1:2-Benzanthracene	5.0 mg.	50	46	44	8	17.4	18.2	161	285
1:2:5:6-Dibenzanthracene	0.02 mg.	50	48	48	28	58.3	58.3	135	329
20-Methylcholanthrene	0.02 mg.	50	45	43	25	55.5	58.1	153	246
Chrysene+1:2-benzanthracene	2.5 mg. of each	50	41	34	15	36.6	44.1	230	346
1:2-Benzanthracene+1:2:5:6-dibenzanthracene	5.0 mg. plus 0.02 mg.	50	39	30	11	28.2	36.6	211	324
1:2:5:6-Dibenzanthracene+20-methylcholanthrene	0.02 mg. of each	50	42	42	33	78.5	78.5	120	180
Tricaprylin controls	0.2 cc. to 2.0 cc.	304	280	233	3	1.1	1.3	268	343
Uninjected controls	None	130	114		0	0.0	0.0		

* Number of survivors at 4 months, when the first tumor appeared among all compounds.

† Number of mice surviving when the first tumor occurred with that particular compound.

‡ Based on the 4-month effective total.

§ Based on the effective total surviving when the first tumor occurred with that compound.

RESULTS

Single carcinogens.—Chrysene and 1:2-benzanthracene were definitely carcinogenic (Table 1). The latter was stronger than the former, as indicated by a higher tumor yield and shorter minimum and average induction periods. Chrysene was a slow carcinogen (Fig. 1). Its carcinogenic index (Iball) was low, being 4.1, in contrast with that of 1:2-benzanthracene, which was 6.4.

The quantity of 1:2:5:6-dibenzanthracene and of 20-methylcholanthrene injected (0.02 mg.) proved, as anticipated, to be near the TD_{50}. Although the eventual tumor yields were the same, the former was a slower carcinogen than the latter (Fig. 1). The induction times of both were shorter than those of the weaker carcinogens.

Neither the data in Table 1 nor those in Figure 1 give adequate information on the rate of nontumor deaths that occurred after the minimum induction time had passed. Table 2 is provided to supply these data, because deaths from intercur-

cent. These controls were all injected with the same specimen of tricaprylin in the same strain of mice, but at intervals over a period of years. One sarcoma was induced with 2 cc. and the other two with 0.5 cc. each. Unfortunately, the oil residues or tumors were not examined spectrophotometrically. The carcinogenic index is negligible, being only 0.37. Tricaprylin cannot be regarded as an inert solvent, since it has a low degree of carcinogenicity comparable to that of some other oily vehicles (50). In view of its comparative excellence as a solvent for carcinogens, it is perhaps not surprising that it alone sometimes attains threshold effects. This concept falls in line with the theory that the difference between tumor-producing and inactive compounds is of a quantitative nature.

Combined carcinogens.—The three combinations of compounds tested are shown in Figure 2. The two milder carcinogens, chrysene and 1:2-benzanthracene, were injected together, using halved doses, or 2.5 mg., of each. Despite the reduced

amount of chemicals, the result was equal to summation of their individual responses at full doses. Two interpretations are possible: (*a*) the dosage of 5 mg. of these compounds was above the saturation dose level for producing the maximum number of tumors. In this case half the amount, or 2.5 mg., would have elicited the same number of tumors. If this explanation is correct, the two chemicals were additive and without mutual interference. (*b*) The result observed was not that of mere summation but of synergism, i.e., an effect greater in combination than the sum of the individual effects. Sufficient data are not at hand to decide whether the observed results were those of summation or of synergism. Further experiments are in progress to clarify these problems and to determine the maximum and minimum dose levels of 1:2-benzanthracene.

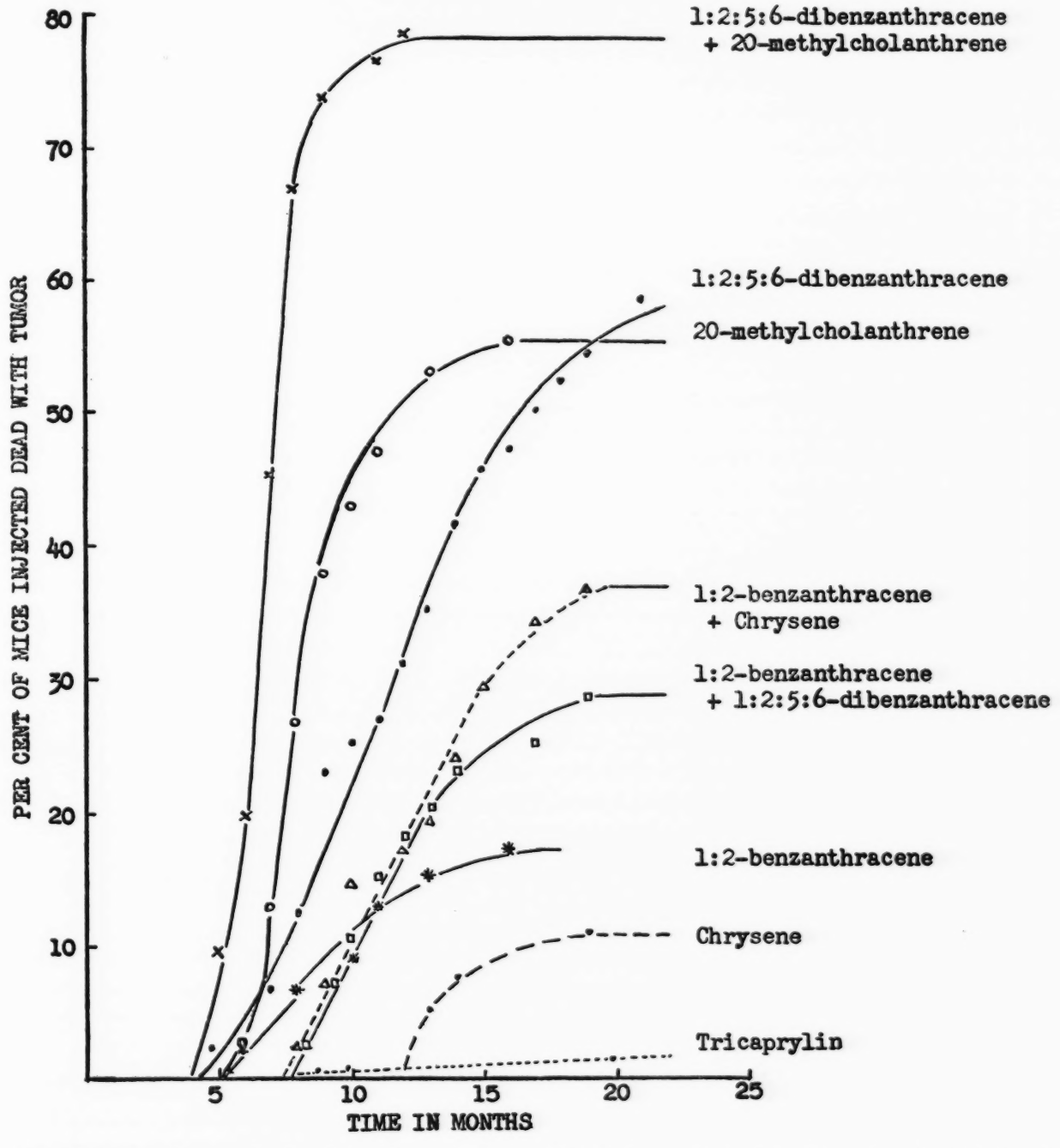

FIG. 1.—Cumulative mortality from induced tumors. Shows cumulative percentage of the mice injected with each compound dying of tumor in each month. For calculating these figures the effective total mice in each experiment was the number surviving at 4 months.

When a weak and a strong carcinogen were injected together (1:2-benzanthracene plus 1:2:5:6-dibenzanthracene), the result was not summation, but apparent inhibition. The tumor yield was about half the sum of their individual tumor yields. This interesting inhibition phenomenon is being further investigated. It has its counterpart in principle (inhibition of a strong by a weak chemical carcinogen of similar molecular configuration), although not with the same compounds and to a smaller degree, in the inhibition of 20-methylcholanthrene by dibenzofluorene, of 1:2:5:6-dibenzanthracene by 1:2:5:6-dibenzacridine, and of 20-methylcholanthrene by chrysene (34, 53). The inhibition was observed on skin-painting by the latter workers, whereas the inhibition observed in the present experiment follows subcutaneous injection.

When the two strong carcinogens were injected together, each in approximately TD_{50}, addition of effects was noted, but not to a full 100 per cent tumor yield. Nevertheless, because the latent period was shortened, the carcinogenic index for the combination equals the sum of the two individual indices. The possible reason for imperfect addition of effects is perhaps found in Table 2, where it is seen that the induction time for these two compounds is different. 1:2:5:6-Dibenzanthracene is slower, so the maximum effects of each on the exposed cells may not have been exerted at the same time.

TABLE 2

RELATION OF TIME OF TUMOR AND NONTUMOR DEATHS TO SURVIVING MICE IN CARCINOGENESIS BY HYDROCARBONS*

Compound tested		Time in months: 0	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21†
Chrysene	No. sarcomas‡	0	0	0	0	0	0	0	0	0	0	2	1	0	0	0	0	1	0	0
	No. surviving mice	50	39	39	37	36	35	32	28	25	24	17	13	11	11	11	11	8	7	6
1:2-Benzanthracene	No. sarcomas	0	0	0	1	0	2	0	1	2	0	1	0	0	1					
	No. surviving mice	50	46	44	38	36	27	25	22	17	11	6	3	1	0					
1:2:5:6-Dibenzanthracene	No. sarcomas	0	0	1	0	2	3	5	1	1	2	2	3	2	1	1	1	1	0	2
	No. surviving mice	50	48	47	47	45	42	37	36	35	33	27	23	18	15	13	12	11	11	9
20-Methylcholanthrene	No. sarcomas	0	0	0	1	5	6	5	2	2	0	3	0	0	1	0	0	0	0	0
	No. surviving mice	50	45	43	42	37	27	20	18	13	9	3	3	3	2	2	2	2	2	2
Chrysene+1:2-Benzanthracene	No. sarcomas	0	0	0	0	0	1	2	3	0	1	2	1	2	0	2	0	1	0	0
	No. surviving mice	50	41	39	38	34	32	26	17	13	11	9	7	5	5	3	3	2	2	2
1:2-Benzanthracene+1:2:5:6-dibenzanthracene	No. sarcomas	0	0	0	0	0	1	2	1	2	1	1	1	0	0	1	0	1	0	0
	No. surviving mice	50	39	35	32	30	27	24	22	16	14	12	11	11	10	9	9	6	6	4
20-Methylcholanthrene+1:2:5:6-dibenzanthracene	No. sarcomas	0	0	4	4	11	9	3	0	1	1	0	0	0	0	0	0	0	0	0
	No. surviving mice	50	42	37	33	22	11	6	5	3	2	2	2	2	2	2	1	1	1	1
Tricaprylin controls	No. sarcomas	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	1	0
	No. surviving mice	304	280	271	264	252	233	219	215	211	207	202	184	178	167	155	142	125	117	68
Uninjected controls	No. surviving mice	130	114	103	91	82	76	65	61	55	48	45	26	25	24	16	16	16	15	

*Nontumor deaths can be calculated by subtracting the surviving mice and deaths due to tumors from the survivors in the preceding month.

† The experiments were terminated early in the 22d month, except for some of the tricaprylin controls.

‡ The sarcomas represent time of death and not induction time.

COMMENTS

The demonstration that 1:2-benzanthracene is carcinogenic requires modifications of several published theories of molecular structure in relation to carcinogenic activity, which had been based on the supposition that this chemical was inactive. For example, Pullman calculated the critical electron density in the K-region of aromatic hydrocarbons and concluded that any value below 1.29e would indicate inactivity (43). The value for 1:2-benzanthracene is 1.283e. The critical level, therefore, must now be revised downward. In addition, the possibility arises that other chemicals within this range might be active and that they should be retested.

The correlation of the absorption spectra of 1:2-benzanthracene and its derivatives with carcinogenic activity was studied by Jones (29). He related the degree of carcinogenic activity to the bathochromic shift of the absorption maximum (*D*), i.e., the difference in wave length between the *D*-peaks in the ultraviolet absorption spectrum of the active carcinogen and the supposed noncarcinogen, 1:2-benzanthracene. Now that 1:2-benzanthracene is shown to be active, the theory requires revision.

Iversen also correlated carcinogenic activity with absorption spectra by using a complex formula (26). He used 1:2-benzanthracene as the base line on the supposition that it was noncarcinogenic. The base line now requires revision.

Several observations in the literature which could not be reconciled with the reported noncarcinogenicity of 1:2-benzanthracene appear to be explained by the present demonstrated activity of this compound. Haddow and Robinson (22) studied the inhibitory effect of aromatic hydrocar-

bons on growth of transplanted tumors. They found that known carcinogens inhibited tumor growth rate but that noncarcinogens did not. They formed a separate category for 1:2-benzanthracene and chrysene, which inhibited growth rate but were thought to belong with the very weak carcinogens. This problem seems to be solved by the results here reported. Demerec (20) observed that 1:2-benzanthracene increased mutation rate in Drosophila, as did three strong carcinogens, whereas other noncarcinogens did not. The inconsistency for 1:2-benzanthracene which he found is eliminated by reclassifying it as a carcinogen according to present results.

1:2-Benzanthracene and chrysene were found to be definite carcinogens, contrary to many reports in the literature. These differences may be explained largely by two factors; namely, the use of a greater number of animals and a longer duration of test in the present work. The minimum induction time for 1:2-benzanthracene in mice was 161 days. Many tests reported in the literature were shorter than that (1, 8, 36, 39, 40, 55), and they may have been negative for that reason. In a few reports, the number of animals tested was probably inadequate as a basis from which to draw conclusions (1, 55). In some reports, the data submitted on survival are inadequate for judging carcinogenicity (24). Only two papers giving negative results appear to be based on reasonably adequate testing. Thus, Boyland and Burrows (10), after subcutaneous injection of aqueous colloidal solutions, had only seven rats alive at 6 months and one at 12 months, all without tumors. Shear and Leiter (48) made subcutaneous injections in mice, ten of which survived for 15 months without tumor. The few tumors previously reported have already been mentioned in the introduction. It can be concluded that the widespread impression that 1:2-benzanthracene is inactive is based on inadequate testing.

The minimum induction time for chrysene in our experiments was 349 days. Many of the reported experiments yielding negative results with chrysene were of much shorter duration than that (23, 31, 32, 37–42), and some of the tests were made on insufficient numbers of animals (33, 37–39). Among those performing more adequate tests were Boyland and Burrows (10), who found no tumors in four rats surviving at 12 months, and Shear and Leiter (48), who had thirteen mice alive without tumor at 12 months. Both these groups had attained the same induction time which we are reporting. The few positive results have already been mentioned in the introduction.

If the present claims for the carcinogenicity of chrysene are conceded, the finding of Haddow and Robinson of the tumor growth-inhibiting action of this compound, like that of strong carcinogens, is clarified (22).

Although 1:2-benzanthracene was found in these experiments to be a more potent tumor-inducing agent than chrysene, this should not be accepted as their final relative position, because they

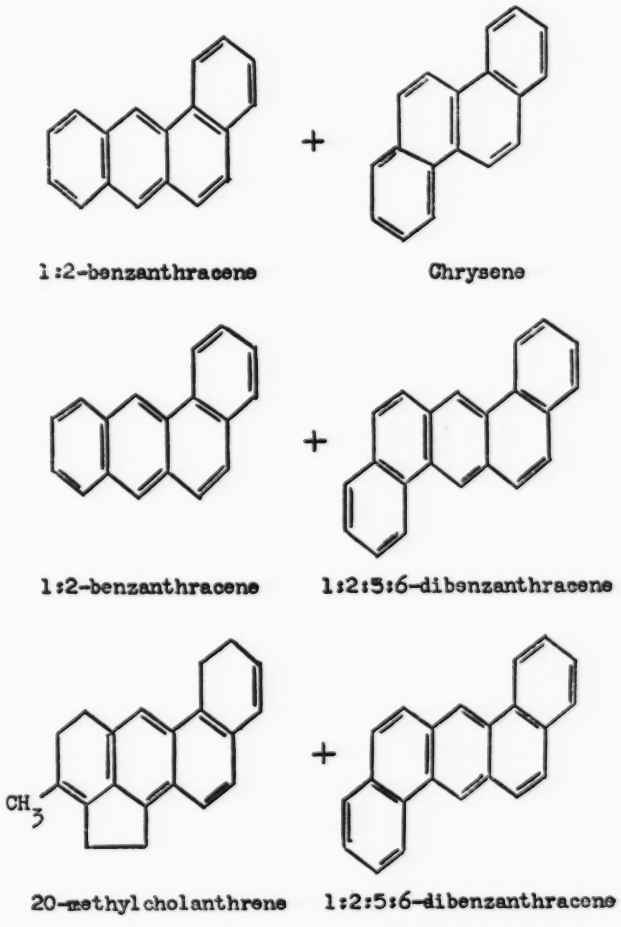

FIG. 2.—Combinations of weak, weak and strong, and strong carcinogenic hydrocarbons tested in the experiment.

were tested in only one solvent. Chrysene had a lower solubility in tricaprylin than did 1:2-benzanthracene. Much of the 5.0 mg. injected was not in solution. It is possible that fewer molecules of the former than of the latter were exerting their effects on the surrounding cells at any one time. The great importance of the solvent on the carcinogenic response is well known. In some other solvent the potency of these two chemicals might be different.

These experiments appear to be the first demonstration of additive or inhibitory carcinogenic effects of chemicals by the parenteral injection method. The previous work had been done by skin-painting or by feeding. The method appears

to open up new and quantitative ways of studying the dose:response relationships and the mechanisms of action.

Neither additive nor synergistic effects of two weak chemical carcinogens have been previously reported. The explanation is difficult, regardless of which phenomenon is accepted as explaining present results. Each chemical presumably was injected in an excessive or supersaturation dose. Just why the tumor yield should have been more than doubled when the dose of chemical was halved is not clear at the moment, although the possibilities were discussed.

The successful summation of the effects of two strong carcinogens was as anticipated and confirms previous results of others by skin-painting.

The inhibition of a strong by a weak carcinogen confirms the work of Lacassagne *et al.* (34) and of Wartman *et al.* (53). Such inhibition has been explained by Crabtree as the competition for the available receptors, presumably the SH groups. This theory might explain the inhibition of 1:2:5:6-dibenzanthracene by 1:2-benzanthracene, but the theory breaks down in the case of the two weaker carcinogens, chrysene and 1:2-benzanthracene, where summation was observed. Further experiments to elucidate this problem are under way.

SUMMARY

1. 1:2-Benzanthracene and chrysene were carcinogenic, inducing sarcoma on subcutaneous injection. The former was stronger than the latter, having both a shorter induction time and a higher tumor yield. The significance of these results with regard to the relation of chemical-physical characteristics of hydrocarbons to their carcinogenicity is discussed.

2. 1:2-Benzanthracene and chrysene injected together showed definite summation of carcinogenic effects if not actually a synergistic action. The tumor yield was greater than the sum of their individual tumor yields, despite the halving of the dose of each compound injected. This observation is significant, because it cannot be explained by the existing theories of inhibition or summation effects of carcinogenic compounds.

3. The carcinogenic effects of 1:2-benzanthracene and 1:2:5:6-dibenzanthracene injected together were not those of summation but apparently of inhibition.

4. The carcinogenic effects of 1:2:5:6-dibenzanthracene and 20-methylcholanthrene injected together were additive, although imperfectly so.

REFERENCES

1. Andervont, H. B., and Shimkin, M. B. Biologic Testing of Carcinogens. II. Pulmonary-Tumor-Induction Technique. J. Nat. Cancer Inst., **1**:225–39, 1940.
2. Bachmann, W. E.; Cook, J. W.; Dansi, A.; de Worms, C. G. M.; Haslewood, G. A. D.; Hewett, C. L.; and Robinson, A. M. Production of Cancer by Pure Hydrocarbons. IV. Proc. Roy. Soc. London, s.B, **123**:343–68, 1937.
3. Barry, G., and Cook, J. W. A Comparison of the Action of Some Polycyclic Aromatic Hydrocarbons in Producing Tumours of Connective Tissue. Am. J. Cancer, **20**:58–69, 1934.
4. Barry, G.; Cook, J. W.; Haslewood, G. A. D.; Hewett, C. L.; Hieger, I.; and Kennaway, E. L. The Production of Cancer by Pure Hydrocarbons. Part III. Proc. Roy. Soc. London, s.B, **117**:318–51, 1935.
5. Berenblum, I. Irritation and Carcinogenesis. Arch. Path., **38**:233–44, 1944.
6. ———. Experimental Inhibition of Tumour Induction by Mustard Gas and Other Compounds. J. Path. & Bact., **40**:549–58, 1935.
7. ———. A System of Grading Carcinogenic Potency. Cancer Research, **5**:561–64, 1945.
8. ———. The Mechanism of Carcinogenesis. A Study of the Significance of Carcinogenic Action and Related Phenomena. *Ibid.*, **1**:807–14, 1941.
9. Bottomley, A. C., and Twort, C. C. The Carcinogenicity of Chrysene and Oleic Acid. Am. J. Cancer, **21**:781–86, 1934.
10. Boyland, E., and Burrows, H. The Experimental Production of Sarcoma in Rats and Mice by a Colloidal Aqueous Solution of 1:2:5:6-Dibenzanthracene. J. Path. & Bact., **41**:231–38, 1935.
11. Bryan, W. R., and Shimkin, M. B. Quantitative Analysis of Dose-Response Data Obtained with Carcinogenic Hydrocarbons. J. Nat. Cancer Inst., **1**:807–33, 1941.
12. ———. Quantitative Analysis of Dose-Response Data Obtained with Three Carcinogenic Hydrocarbons in Strain C3H Male Mice. *Ibid.*, **3**:503–31, 1943.
13. Carruthers, C. Influence of Heptaldehyde on Carcinogenic Action of Methylcholanthrene. Arch. Path., **30**:1184–91, 1940.
14. Cook, J. W. The Production of Cancer by Pure Chemical Compounds. Cong. internat. de lucha cient. y soc. contra el cancer, Madrid, **2**:373–78, 1933.
15. Crabtree, H. G. Retardation of the Rate of Tumor Induction by Hydrolyzing Chlor-Compounds. Cancer Research, **1**:39–43, 1941.
16. ———. Retardation of Rate of Tumor Induction by Substances Which Inhibit Glycolysis. J. Path. & Bact., **51**:303–9, 1940.
17. ———. Influence of Unsaturated Dibasic Acids on the Induction of Skin Tumors by Chemical Carcinogens. Cancer Research, **5**:346–51, 1945.
18. ———. Influence of Bromobenzene on the Induction of Skin Tumors by 3:4-Benzpyrene. Cancer Research, **4**:688–93, 1944.
19. ———. Some Effects of Aromatic Hydrocarbons on Sulfur Metabolism and Tumor Induction in Mice. *Ibid.*, **6**:553–59, 1946.
20. Demerec, M. Mutations Induced by Carcinogens. Brit. J. Cancer, **2**:114–17, 1948.
21. Haddow, A. Mode of Action of Chemical Carcinogens. Brit. M. Bull., **4**:331–42, 1947.
22. Haddow, A., and Robinson, A. M. The influence of Various Polycylic Hydrocarbons on the Growth Rate of Transplantable Tumours. Proc. Roy. Soc. London, s.B, **122**:442–76, 1937.
23. Haddow, A.; Scott, C. M.; and Scott, J. D. Influence of Certain Carcinogenic and Other Hydrocarbons on Body

Growth in the Rat. Proc. Roy. Soc. London, s.B, **122**: 477–507, 1937.

24. HIEGER, I. On the Mechanism of Carcinogenesis by Chemical Compounds. Am. J. Cancer, **28**:522–29, 1936.
25. IBALL, J. The Relative Potency of Carcinogenic Compounds. Am. J. Cancer, **35**:188–90, 1939.
26. IVERSEN, S. A Possible Correlation between Absorption Spectra and Carcinogenicity. Copenhagen: Einar Munksgaard, 1949.
27. JAFFÉ, W. G. The Response of Rats to the Simultaneous Application of Two Different Carcinogenic Agents. Cancer Research, **7**:113–16, 1947.
28. ———. The Response of Mice to the Simultaneous Application of Two Different Carcinogenic Agents. *Ibid.*, pp. 529–30, 1947.
29. JONES, R. N. The Ultraviolet Absorption Spectra of Some Derivatives of 1:2-Benzanthracene. J. Am. Chem. Soc., **62**:148–52, 1940.
30. KENNAWAY, E. L. Further Experiments on Cancer-producing Substances. Biochem. J., **24**:497–504, 1930.
31. ———. Cancer-producing Tars and Tar Fractions. J. Indust. Hyg., **5**:462–88, 1924.
32. ———. The Formation of a Cancer-producing Substance from Isoprene. J. Path. & Bact., **27**:233–38, 1924.
33. ———. On the Cancer-producing Factor in Tar. Brit. M. J., **1**:564–67, 1924.
34. LACASSAGNE, A.; BUÜ-HOI; and RUDALI, G. Inhibition of the Carcinogenic Action Produced by a Weakly Carcinogenic Hydrocarbon on a Highly Active Carcinogenic Hydrocarbon. Brit. J. Exper. Path., **26**:5–12, 1945.
35. LAVIK, P. S.; MOORE, P. R.; RUSCH, H. P.; and BAUMANN, C. A. Some Additive Effects of Carcinogenic Hydrocarbons. Cancer Research, **2**:189–92, 1942.
36. LEES, J. C. The Inhibition of Growth by 1:2:5:6-Dibenzanthracene and Other Agents. Quart. J. Exper. Physiol., **27**:161–70, 1937.
37. NOBLE, R. L. Effect of Synthetic Oestrogenic Substances on the Body Growth and Endocrine Organs of the Rat. Lancet, **235**:192–95, 1938.
38. ———. Effects of Synthetic Oestrogens and Carcinogens when Administered to Rats by Subcutaneous Implantation of Crystals or Tablets. J. Endocrinol., **1**:216–29, 1939.
39. ORR, J. W. The Changes Antecedent to Tumor Formation during the Treatment of Mouse Skin with Carcinogenic Hydrocarbons. J. Path. & Bact., **46**:495–515, 1938.
40. ———. An Investigation of the Histological Changes in the Subcutaneous Tissues of Mice during the Induction of Sarcoma by Carcinogenic Hydrocarbons. *Ibid.*, **49**:157–70, 1939.
41. POLLIA, J. A. Investigations on the Possible Carcinogenic Effect of Anthracene and Chrysene and Some of Their Compounds. I. The Effect of Painting on the Skin of Mice. J. Indust. Hyg. & Toxicol., **21**:[illegible] 1939.
42. ———. Investigations on the Possible Carcinogenic Effect of Anthracene and Chrysene and Some of Their Compounds. II. The Effects of Sucbutaneous Injection in Rats. *Ibid.*, **23**:449–51, 1941.
43. PULLMAN, A. Structure électronique et pouvoir cancérigène des hydrocarbures aromatiques condensés. Bull. Assoc. franç. p. l'étude du cancer, **33**:120–30, 1946.
44. RILEY, J. F., and WALLACE, A. B. Stimulation and Inhibition of Connective Tissue in Mice Following Injection of Benzpyrene. Brit. J. Exper. Path., **22**:24–28, 1941.
45. RILEY, J. F., and PETTIGREW, F. Retarding Effect of Glyceraldehyde on Benzpyrene Sarcoma Formation in Mice. Cancer Research, **4**:502–4, 1944.
46. RUSCH, H. P.; KLINE, B. E.; and BAUMANN, C. A. The Nonadditive Effect of Ultraviolet Light and Other Carcinogenic Procedures. Cancer Research, **2**:183–88, 1942.
47. SCHÜRCH, O., and WINTERSTEIN, A. Über die krebserregende Wirkung aromatischer Kohlenwasserstoffe. Ztschr. f. physiol. Chem., **236**:79–91, 1935.
48. SHEAR, M. J., and LEITER, J. Studies in Carcinogenesis. XVI. Production of Subcutaneous Tumors in Mice by Miscellaneous Polycyclic Compounds. J. Nat. Cancer Inst., **2**:241–58, 1941.
49. STASNEY, J.; PASCHKIS, K. E.; CANTAROW, A.; and ROTHENBERG, M. S., Neoplasms in Rats with 2-Acetaminofluorene and Sex Hormones. II. Cancer Research, **7**:356–62, 1947.
50. STEINER, P. E.; STEELE, R.; and KOCH, F. C. The Possible Carcinogenicity of Overcooked Meats, Heated Cholesterol, Acrolein, and Heated Sesame Oil. Cancer Research, **3**: 100–107, 1943.
51. TWORT, C. C., and FULTON, J. D. Further Experiments on the Carcinogenicity of Synthetic Tars and Their Fractions. J. Path. & Bact., **33**:119–43, 1930.
52. TWORT, J. M., and TWORT, C. C. Comparative Activity of Some Carcinogenic Hydrocarbons. Am. J. Cancer, **35**:80–85, 1939.
53. WARTMAN, W. B.; SHUBIK, P.; HILL, W. T.; REEB, B. B.; STANGER, D. W.; and RIEGEL, B. Delay of Methylcholanthrene Carcinogenesis by 1,2,5,6-Dibenzofluorene. Cancer Research, **10**:247, 1950.
54. WHITE, F. R., and ESCHENBRENNER, A. B. Note on the Occurrence of Hepatomas in Rats Following the Ingestion of 1:2-Benzanthracene. J. Nat. Cancer Inst., **6**:19–21, 1945.
55. WOLFE, J. M., and BRYAN, W. R. Effects Induced in Pregnant Rats by Injection of Chemically Pure Carcinogenic Agents. Am. J. Cancer, **36**:359–68, 1939.

The Cheek Pouch of the Hamster as a Site for the Transplantation of a Methylcholanthrene-induced Sarcoma*

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INTRODUCTION

A method by which neoplastic tissue may be grown in a physiological environment and at the same time be readily accessible for periodic experimentation and observation is described in this paper. The membranous cheek pouch of the hamster (*Mesocricetus auratus*), used by Fulton, Jackson, and Lutz (5) for the cinephotomicrographic study of small blood vessels, was selected as the site for the transplantation of a methylcholanthrene-induced hamster sarcoma. The hamster has been used relatively little in tumor work. Few inbred strains have been developed, and the only report of a spontaneous tumor in the hamster has been by Ashbel (3), who found thirteen cases in 1,000 animals over a period of 7 years. Gye and Foulds (6) and Halberstaedter (7) have produced benzpyrene-induced tumors in hamsters. Crabb (4) described a transplantable sarcoma, induced in hamsters with 9,10-dimethyl-1,2-benzanthracene, and in 1949 reported the relation of duration of a transplanted sarcoma to metastasis. Kelsall (8) described hematopoiesis in the spleen of tumor-bearing hamsters.

Subpannicular induction and transplantation have been commonly practiced. Crabb (4) obtained 100 per cent takes when he transferred his benzanthracene-induced sarcoma by means of a hypodermic needle to this site. When the object is to study the effect of the tumor on the animal, this site is useful. When the object is to study the tumor *per se*, or the local tissue responses, subpannicular growth has obvious disadvantages. The mouse skin-flap preparation described by Algire (1) and modified by Algire and Legallais (2) has become virtually a tissue culture *in vivo* preparation. It has, however, definite limitations, imposed by the necessary operative procedures and by the physical confines of the chamber.

The advantages realized by using the membranous cheek pouch of the hamster as a site for transplantation are (*a*) the tumor grows freely without physical hindrance and in a normal physiological environment; (*b*) the same tumor can be observed at successive stages (measured, photographed); (*c*) the tumor may be subjected to various experimental procedures (chemical and physical) with no undesirable disturbance beyond that produced by anesthesia; and (*d*) transillumination for microscopic study of early stages of growth and vascularization is practicable.

METHOD

Hamsters of both sexes, 8–10 weeks old and weighing 90–100 gm., were used throughout this work. The original tumor was induced in hamsters by a series of six weekly subpannicular injections of 0.25 cc. of 0.4 per cent methylcholanthrene in oil of lard. This tumor is tentatively classified as a type of spindle-cell sarcoma, histologically somewhat different from the benzanthracene-induced sarcoma described by Crabb (4), although no pictures of his original tumor were published. The methylcholanthrene-induced tumor was propagated by transplantation in the flank of other hamsters and in cheek pouches.

This material was the source of the 35 cheek pouch transplants, the growth characteristics of which are reported here. Pieces of hamster tissue such as liver, kidney, adrenal cortex, cheek pouch, epidermis, and skeletal muscle were used to compare the effect of normal tissue *per se* with that of the neoplastic tissue. A few minutes before making a cheek pouch transplant, the hamster was anesthetized by an intraperitoneal injection of veterinary nembutal (Abbott) in a dose of 0.1 cc/100 gm body weight. The animal was placed in a spun-steel preparation dish, developed for blood

* This work was supported, in part, by a grant from the American Cancer Society (Massachusetts Division).

Received for publication September 3, 1950.

vessel work (9), the cheek pouch gently everted with forceps, and held with "bank" pins over the optical glass block. With fine scissors, an incision about 1 mm. long was made through the epidermal layer. A piece of tumor or control tissue about 1 c. mm. was inserted with forceps into the loose connective tissue beneath the epidermis and then massaged gently 5 mm. beyond the edge of the incision. When the pins were removed the incision closed by itself. Instruments were clean, but no special surgical asepsis was necessary. No infection occurred either at the incision or at the points of pinning.

The three dimensions of the tumor transplant were measured periodically by means of an ocular micrometer and a vertical scale attached to a binocular microscope (×9). The symmetrical form assumed by the free-growing tumor permitted a reasonably accurate determination of its volume.

Tissues from the original induced tumor and from cheek pouch transplants taken for microscopic study were fixed in 4 per cent formol or in Zenker's fluid and stained with hematoxylin and eosin, or with phloxine.

RESULTS

Subpannicular injections of methylcholanthrene into nine hamsters resulted in the induction of tumors in five. Two tumors were palpable in 8 weeks, one in 16 weeks, one in 23 weeks, and one in 28 weeks. The tumor-bearing animals were used as donors, and no data were obtained concerning the malignancy of their tumors at this stage. Thirty-five transplants of the methylcholanthrene-induced tumor were made, and all became successfully established. Twenty-nine of these were derived from the same original induced tumor, at the same time and under the same conditions. These were used to compare the effects of the number of passages on the rate of growth of transplants. Six were first generation transplants (directly from the original tumor), nineteen were second generation (one passage), and four were third generation. The average length of time for all transplants from transplantation to the first measurable increase in size was 4 days. During this time vascularization appeared to be established. In the next 10 days the average transplant increased gradually from 2.6 c. mm. to 60 c. mm. After the fourteenth day growth was exceedingly rapid. Thus, the average tumor had grown to 445 c. mm. in the next 10 days. Some tumors became ulcerated at this time. Examination of these tumors showed a large central necrotic region and a thin cortical layer of active growth. Others continued to increase in size at a uniform rate, the largest becoming 2,380 c. mm. at the end of 43 days. The time at which ulceration occurred varied between 24 and 43 days from transplantation. After ulceration occurred, the size was difficult to determine, but the total volume increase was definitely retarded.

The data indicate that each passage was followed by an increased rate of growth of the transplant (Chart 1). After 18 days the average size of

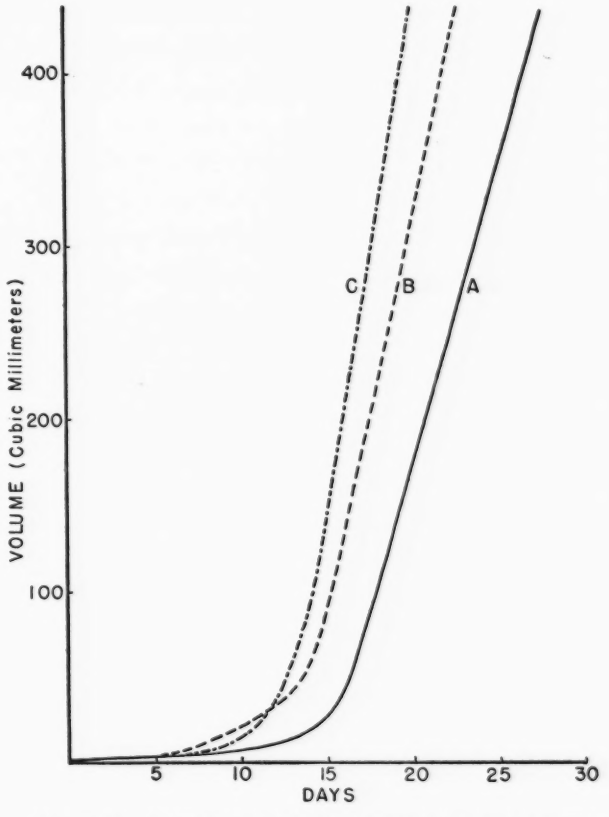


CHART 1.—Growth of methylcholanthrene-induced tumor transplants in the cheek pouch. *A*, transplants from the original subpannicular tumor. *B*, transplants after one passage through the cheek pouch. *C*, transplants after two passages through the cheek pouch.

first generation transplants in six animals was 105 c. mm. (Chart 1, line A). At the same time the average size of second generation transplants in nineteen animals was 222 c. mm. (line B), and the average size of third generation transplants in four animals was 316 c. mm. (line C).

Microscopically, the original methylcholanthrene-induced tumor appears to be a spindle-cell sarcoma type with areas of pleomorphism (Figs. 1–3). In the spindle-cell regions the cells are close together with little if any collagen, being arranged typically in bundles of different sizes, which run through the tumor in various directions. The nuclei are elongated and vesicular. The tumor is richly vascular, with small arteries and capillaries as well as typical vascular slits. Often nodules pro-

ject into the vascular sinuses. In the pleomorphic regions many types of cells are found, including multinucleated giant cells. Little if any supporting substance is evident. The nuclei show a great range of shape, size, and density, and mitotic figures are numerous. Naked vascular sinuses are abundant. Foci of necrosis are seen in deeper regions of the tumor.

The cheek pouch transplants were generally symmetrical in shape, oval, or spherical (Figs. 5–6). As they increased in size, active growth was confined to a definite cortical region, while the entire central portion became necrotic. The histology of the cheek pouch transplants is similar to the original tumor (Fig. 4). The tumor mass seems to be composed largely of anaplastic cells of pleomorphic nature. Those on the peripheral growth area, contiguous with the connective tissue, tend to be spindle-shaped, with abundant cytoplasm and large nuclei. They are arranged loosely, in bundles at places, although no definite capsule is present. In some peripheral portions no spindle-cells are evident. In these actively growing regions the cells tend to become spherical, and mitotic figures are numerous. Small blood vessels with a definite vascular lining are numerous but largely confined to the outer region of the tumor, while others more deeply situated toward the necrotic region are less numerous and irregularly sinusoidal in nature, with and without vascular lining.

Metastases from the methylcholanthrene tumor were found in the liver, lung, spleen, mesentery, and intestinal wall (Figs. 7–10). Microscopically, the spleen metastasis resembles the original tumor more clearly than it does the cheek pouch transplant, showing a preponderance of spindle-cells, arranged in bundles. It is moderately well vascularized, but no vascular slits are found. Some round cells are highly anaplastic, and mitotic figures are common. The cells of the liver metastasis show considerable pleomorphism, with little tendency for them to be arranged in bundles. They are exceedingly anaplastic with many multinucleated cells, some of giant size. Mitotic figures are common. A few small foci of necrosis are found. The same variety of cells is present as occurs in the original tumor. A few small blood vessels are present. The periphery shows invasion of the tumor cells among the cords of the liver (Fig. 7). The intestinal metastasis was spherical, with central cells, which were anaplastic and pleomorphic, and a cortical layer of spindle cells (Fig. 10).

SUMMARY

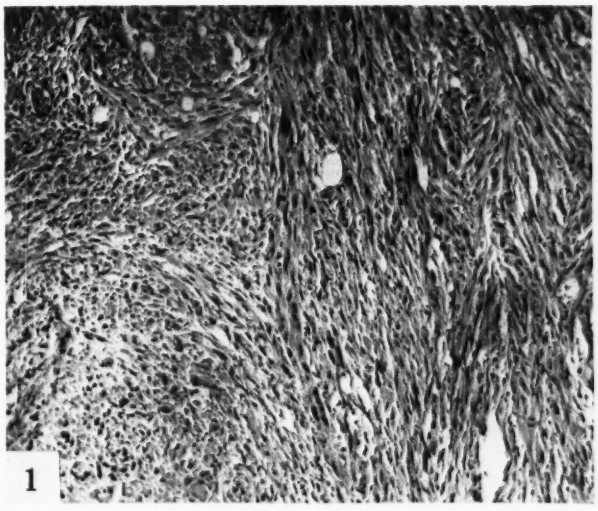
The cheek pouch of the hamster has been used as a site for the transplantation of a new methylcholanthrene-induced malignant sarcoma. The advantages of this site are (*a*) the tumor grows freely without physical hindrance; (*b*) the same tumor can be observed frequently at successive stages (measured, photographed); (*c*) the tumor may be subjected to various experimental procedures with little disturbance of the physiological environment; (*d*) a high percentage of "takes" is obtained; and (*e*) transillumination for microscopic study of early stages of growth and vascularization is practicable.

The original tumor was induced in five hamsters out of nine. Thirty-five cheek pouch transplants were made, and all became successfully established, measurable, on the average, in 4 days. The tumors were either spheroid or ovoid. Growth was rapid and, after the fourteenth day, essentially exponential. Ulceration usually occurred between the twenty-fourth and the forty-third day. Each successive transplant increased in rate of growth.

Microscopically, the original methylcholanthrene-induced tumor appeared to be a spindle-cell sarcoma with areas of pleomorphism. The cheek pouch transplants were similar but with more anaplasia and pleomorphism. Metastases were found in the liver, lung, spleen, mesentery, and intestinal wall. Similar anaplastic and pleomorphic characteristics were found in the metastases. While that in the spleen showed some tendency for spindle cells to be arranged in bundles, the liver metastasis was extremely anaplastic and pleomorphic.

REFERENCES

1. Algire, G. H. An Adaptation of the Transparent Chamber Technique to the Mouse. J. Nat. Cancer Inst., **4**:1–11, 1943.
2. Algire, G. H., and Legallais, F. Y. Recent Developments in the Transparent-Chamber Technique as Adapted to the Mouse. J. Nat. Cancer Inst., **10**:225–53, 1949.
3. Ashbel, R. Spontaneous Transmissible Tumors in the Syrian Hamster. Nature, **155**:607, 1945.
4. Crabb, E. D. Transplantable 9,10-Dimethyl-1,2-benzanthracene Sarcoma in the Syrian Hamster. Cancer Research, **6**:627–36, 1946.
5. Fulton, G. P.; Jackson, R. G.; and Lutz, B. R. Cinephotomicroscopy of Normal Blood Circulation in the Cheek Pouch of the Hamster. Science, **105**:361, 1947.
6. Gye, W. E., and Foulds, L. A Note on the Production of Sarcomata in Hamsters by 3,4-Benzyprene. Am. J. Cancer, **35**:108, 1939.
7. Halberstaedter, L. Tumours Produced in Hamsters by Benzpyrene. Am. J. Cancer, **38**:351–58, 1940.
8. Kelsall, M. A. Hematopoiesis in the Spleen of Tumor-bearing Hamsters. J. Nat. Cancer Inst., **10**:625–29, 1949.
9. Lutz, B. R.; Fulton, G. P.; and Akers, R. P. The Neuromotor Mechanism of the Small Blood Vessels in Membranes of the Frog (*Rana pipiens*) and the Hamster (*Mesocricetus auratus*) with Reference to the Normal and Pathological Conditions of Blood Flow. Exper. Med. & Surg., **8**:258–87, 1950.



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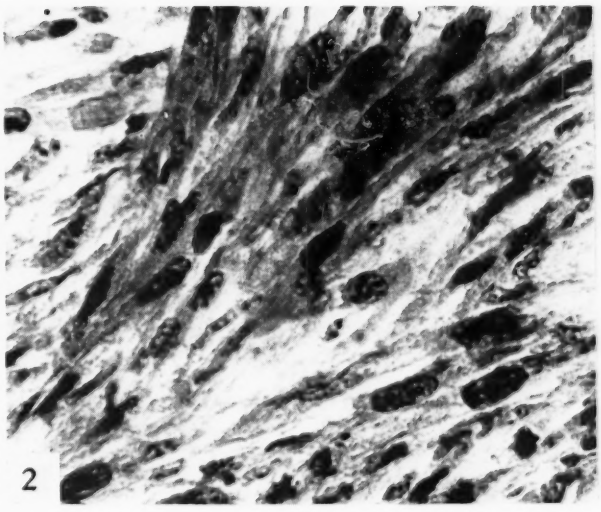

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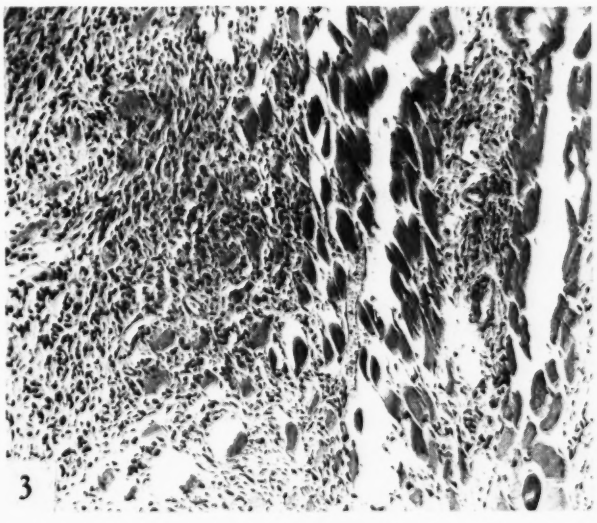

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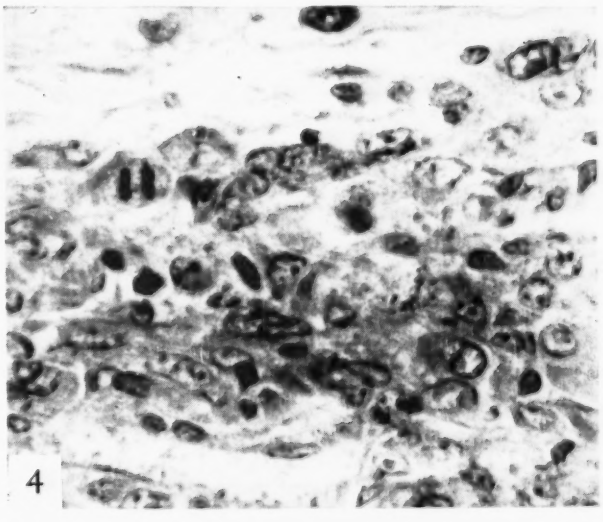

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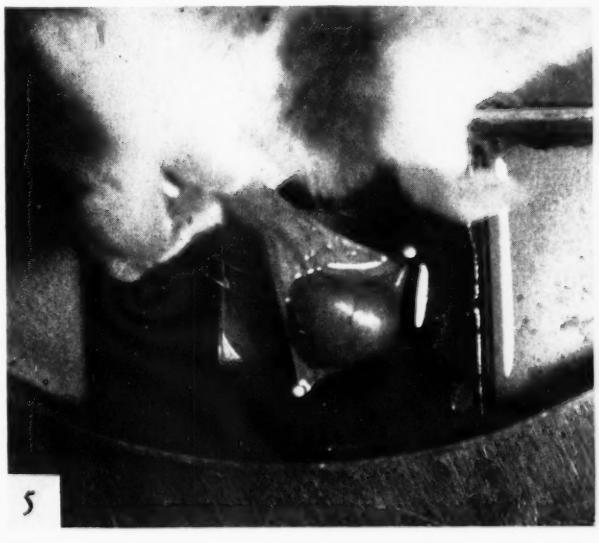

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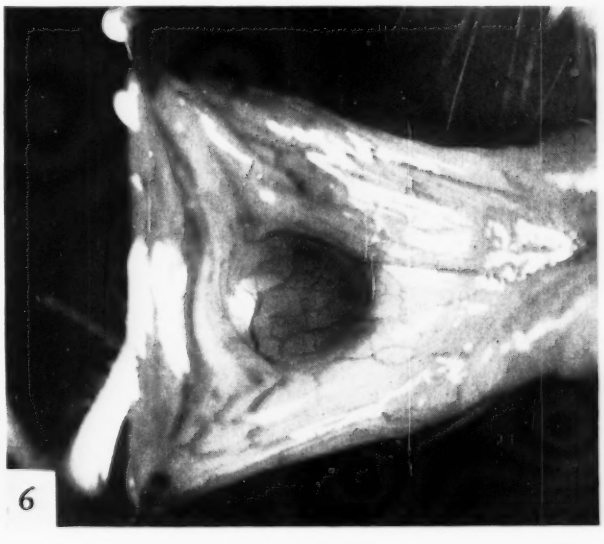

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FIG. 1.—Methylcholanthrene-induced tumor produced in the hamster by subpannicular injection. Zenker's solution, phloxine, and methylene blue. Mag. ×150.

FIG. 2.—Original induced tumor showing spindle-cell nature. Mag. ×645.

FIG. 3.—Original induced tumor invading skeletal muscle. Mag. ×150.

FIG. 4.—Methylcholanthrene cheek pouch transplant, outer growth zone showing pleomorphism and anaplasia. Zenker's solution, phloxine and methylene blue. Mag. ×645.

FIG. 5.—Cheek pouch transplant of methylcholanthrene tumor, 40 days old, volume 1,125 c. mm.

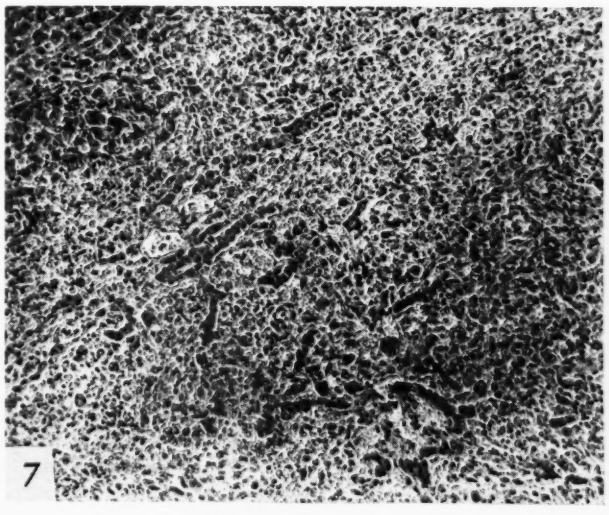
FIG. 6.—Methylcholanthrene cheek pouch transplant, 16 days old, volume 99 c. mm. Note vascularization.

FIG. 7.—Metastasis in liver from methylcholanthrene cheek pouch transplant after 107 days. Note remaining cords of liver cells. Mag. ×150.

FIG. 8.—Portion of liver metastasis shown in Figure 8. One or two liver cells remaining. Note resemblance to cheek pouch transplant shown in Figure 9. Mag. ×645.

FIG. 9.—Metastasis in spleen from cheek pouch transplant after 107 days. Hematoxylin and eosin. Mag. ×430.

FIG. 10.—Metastasis in wall of small intestines from cheek pouch transplant after 107 days. Note the pleomorphism. One of several hundred pearl-like nodules in the peritoneum and mesentery. Mag. ×645.



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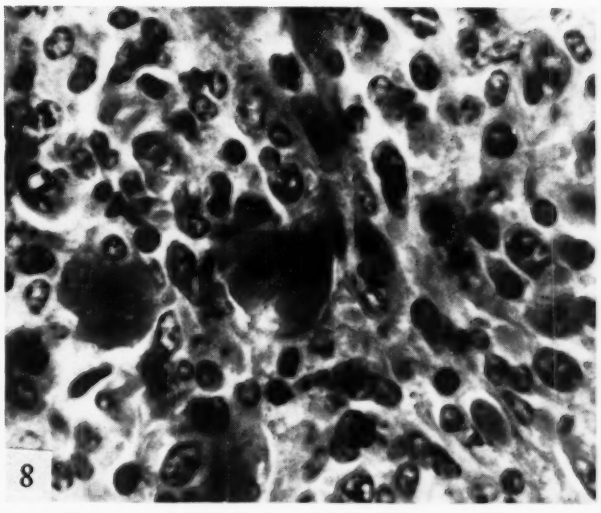

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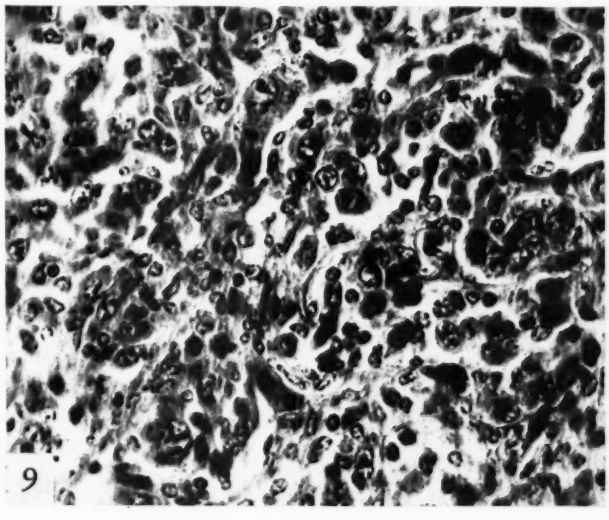

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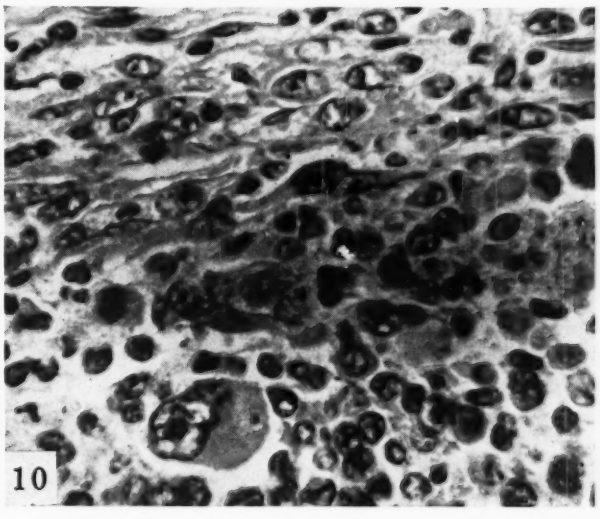

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The Amino Acid Content of Certain Normal and Neoplastic Tissues*

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An understandable interest in the composition of tumor protein has led to a fairly extensive literature (26, 4) on the amounts of amino acids found in tumors, although the methods of analysis used have not always been reliable and the results at best are fragmentary. However, the development of microbiological methods for determining individual amino acids now makes it possible to ascertain the amino acid composition of tumors, and Dunn *et al.* (4) have recently published the percentages of twelve amino acids in a transplantable fibrosarcoma grown in the Long-Evans strain of rats. The present survey deals with the amounts of eighteen amino acids in fifteen different samples of normal and neoplastic tissues.

METHODS

The samples analyzed are listed in Table 1. The so-called control tissues included muscle and liver from normal rats, as well as samples of nontumorous liver from rats that had been fed a diet low in calories or a noncarcinogenic azo dye, *p*-aminoazobenzene (AAB). A sample of cirrhotic liver from a rat fed *m'*-methyl-*p*-dimethylaminoazobenzene (*m'*DAB) was included, as well as a nontumorous lobe from the liver of a rat that had a large hepatoma due to *m'*DAB. The sample of rat tumors analyzed included the transplantable Flexner-Jobling carcinoma, a primary sarcoma induced by methylcholanthrene (MC), hepatomas due to *p*-dimethylaminoazobenzene (DAB) and *m'*DAB, respectively, and a spontaneous mammary fibrosarcoma.

* Published with the permission of the Director of the Wisconsin State Agricultural Experiment Station. Supported in part by the Jonathan Bowman Cancer Fund and by the American Cancer Society through the Committee on Growth. Further details are recorded in a Ph.D. thesis entitled "Amino Acid Metabolism in Normal and Deficient Rats and Mice," H. E. Sauberlich, University of Wisconsin, July 1948. Presented in part to the American Association for Cancer Research, Detroit, April 1949. Mr. C. E. Blades assisted with certain of the determinations.

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Received for publication September 13, 1950.

The samples were weighed fresh, dried to constant weight in a vacuum oven at 55°–60° C. and 20 mm. Hg, and extracted with anhydrous ether. The nitrogen content of the fat-free dry residues was determined by a semi-micro Kjeldahl method. Aliquots of the dried residue were then hydrolyzed (24) with 4 N HCl or NaOH; approximately 20 mg. of sample was placed in a Pyrex test tube with 10 ml. of the acid or alkali; and the sealed tube was autoclaved at a pressure of 15 lbs. In a preliminary series, all samples were hydrolyzed for 6 hours. In a second series, all samples were hydrolyzed for 3, 5, 8, 12, 18, and 24 hours. Samples of casein, of casein plus known amounts of amino acids, and of mammary tumor plus known amounts of the amino acids were also hydrolyzed and analyzed. The samples were neutralized and stored in the cold under toluene. Suitable dilutions were made from these "master solutions" for direct use in the microbiological assays.

Sixteen of the amino acids were determined in the acid hydrolyzates with *Leuconostoc mesenteroides* P-60, *Leuconostoc citrovorum* 8081, and *Streptococcus faecalis*, as described previously (24, 19–20); tryptophan and tyrosine were determined in the alkaline hydrolyzates. The results were expressed in the conventional manner as the percentage of the amino acid in the fat-free dry residue corrected to 16 per cent N; this approximates the percentage of amino acid in the total tumor protein. For most of the amino acids in most of the samples, maximum values were attained after 5–8 hours of hydrolysis, and the values after 3 or 12 hours were not significantly lower. As hydrolysis was prolonged, however (18), the apparent amino acid content sometimes decreased. With a few exceptions the results presented are the averages for the 5- and 8-hour hydrolyzates.

RESULTS

Each of the eighteen amino acids was found to be present in all the tumors examined; and for four of the tumors—the Flexner-Jobling, a sarcoma due to methylcholanthrene, and the hepatomas

due to DAB and *m*'DAB—the relative amounts of the amino acids were very similar from tumor to tumor (Group II, Tables 2 and 3). The most abundant amino acids in these tumors were glutamic acid, 11.7–13.3 per cent; aspartic acid, 8.4–9.1 per cent; leucine, 7.2–8.9 per cent; and lysine, 6.5–8.3 per cent. The least abundant were tryptophan, 0.9–1.2 per cent, and methionine, 1.9–2.0 per cent. All other amino acids were present in intermediate amounts. The apparent differences in the amino acid content of the different tumors were no greater than those observed among different samples of the same tumor (4, 18). The values for the amino acids in these four tumors also agreed well with those of Dunn *et al.* (4) for a fibrosarcoma (Table 4).

The amino acid composition of the four representative tumors was very similar to that of the normal and pre-neoplastic rat tissues analyzed (Group I, Tables 2 and 3). In this connection, the results for arginine were representative of those for all the other amino acids: 5.4–6.2 per cent in the four tumors as compared with 5.0–6.0 per cent in the "control" tissues of the rat.

Extensive studies by others on the amino acid content of various types of meat have indicated a very general similarity in the amounts of the amino acids, not only in the various cuts of meat, but even among the various kinds of meat such as pork, beef, or mutton (1, 5–6, 8, 12, 14, 21). The present studies indicate that normal rat tissues, as well as rat tumors, resemble ordinary meats in amino acid content (Table 4).

The two specimens of mammary fibrosarcoma analyzed differed from the other tumors in that the content of alanine and arginine was increased significantly, that of proline was doubled, and that of glycine was trebled to an average of 17.1 per cent of the total protein present (Tables 2 and 3). The percentages of many other amino acids were less in the mammary tumor than in the other tumors analyzed, viz., aspartic acid, histidine, isoleucine, leucine, lysine, methionine, threonine, tyrosine, valine, and especially tryptophan. The composition of the mammary tumor therefore resembled the composition of collagen or gelatin (Table 4) more closely than that of the other tumors analyzed, suggesting that the mammary tumor consisted of ordinary tumor protein diluted with much connective tissue.

TABLE 1

FAT, MOISTURE, AND NITROGEN CONTENT OF CERTAIN NORMAL AND NEOPLASTIC TISSUES

Group	Dry matter	Fat in solids (per cent)	N in fat-free dry residue
I. Control tissues			
Rat muscle	23	3.7	15.0
" liver	31.2	8.7	11.6
" " , 50 per cent cals.	33.4	9.7	13.2
" " , AAB	32.0	13.3	12.3
" " , *m*'DAB, non-T. lobe	26.9	11.2	13.2
" " , *m*'DAB, cirrhosis	26.6	18.4	13.0
II. Rat tumors			
Flexner-Jobling carc.	17.3	25.4	14.1
Sarcoma MC subcu.	13.1	8.2	13.3
Hepatoma DAB	15.8	15.8	13.8
" *m*'DAB	16.6	9.4	13.3
III. Rat spont. mammary adenocarc.	21.5	6.5	15.3
IV. Miscellaneous			
Rabbit spont. skin tumor	17.4	5.9	11.7
Human tumor of colon	18.4	10.5	13.9
" colon tissue	25.1	42.4	13.5

DISCUSSION

The significance of any study can be no greater than the reliability of the analytical methods employed. The recoveries of amino acids added prior to hydrolysis were the same for tumors as for normal tissue, and both were similar to those reported in studies on other natural materials (6, 8, 17–20, 24–25). Nevertheless, it must be recognized that errors are still inherent in certain of the procedures used. The hydrolysis of protein in the presence of carbohydrates can result in low values for lysine, arginine, and tryptophan (9, 11, 13, 16). Losses of cystine during hydrolysis are probable (7, 15). The determination of glycine involves the use of standard curves in which the blank is high (24). On the other hand, microbiological assays have been used widely in the determination of amino acids in meats, foodstuffs, blood, and urine, and where the available chemical methods are sufficiently sensitive and reliable, the correspondence between chemical and microbiological results has usually been good (2, 8, 23, 25).

TABLE 2

AMINO ACID CONTENT OF CERTAIN NORMAL AND NEOPLASTIC TISSUES

(Percentages of fat-free, moisture-free tissue calculated to 16 per cent N)

Group	Alanine	Arginine	Aspartic acid	Cystine	Glutamic acid	Glycine	Histidine	Isoleucine	Leucine
					(per cent)				
I. Controls									
Rat muscle	7.5	6.0	8.6	2.0	15.0	5.6	2.0	5.2	7.7
" liver	4.7	5.0	7.6	1.6	12.3	5.4	2.0	4.9	8.2
" " , 50 per cent cals.	6.7	5.8	8.8	1.3	12.1	5.9	2.4	5.1	9.1
Rat liver, AAB	6.3	5.3	8.6	1.0	10.4	5.6	2.2	4.8	8.9
" " , *m*'DAB non-T. lobe	6.5	5.7	8.5	1.8	11.3	4.9	2.2	4.9	8.5
Rat liver, *m*'DAB, cirrhosis	5.3	5.7	8.6	1.5	11.5	6.1	2.2	4.8	7.3
II. Rat tumors									
Flexner-Jobling carc.	6.4	5.8	8.8	1.1	11.7	5.2	1.9	5.3	7.9
Sarcoma MC subcu.	7.4	5.4	8.3	2.2	12.2	5.0	2.2	5.0	8.9
Hepatoma DAB	7.6	5.5	9.1	2.1	13.3	5.1	2.7	5.6	7.2
" *m*'DAB	6.5	6.2	8.7	2.0	12.3	6.3	2.1	4.3	7.5
III. Rat spont. mammary fibrosarcoma	8.0	6.8	6.7	1.9	10.2	17.1	1.2	2.5	4.7
IV. Miscellaneous									
Rabbit spont. skin T.	5.9	6.2	7.4	1.3	10.3	8.0	1.6	3.7	6.3
Human T. of colon	7.8	6.6	7.5	1.0	12.8	11.0	2.0	4.3	7.8
" colon tissue	6.3	6.2	8.3	1.8	11.8	8.0	2.1	5.0	8.1

TABLE 3

AMINO ACID CONTENT OF CERTAIN NORMAL AND NEOPLASTIC TISSUES

(Percentages of fat-free, moisture-free tissue calculated to 16 per cent N)

Group	Lysine	Methionine	Phenylalanine	Proline	Serine	Threonine	Tryptophan	Tyrosine	Valine
					(per cent)				
I. Controls									
Rat muscle	8.3	2.5	3.6	4.1	3.9	4.1	1.2	3.1	4.3
" liver	5.2	2.2	4.3	4.1	4.7	3.8	1.3	3.6	5.2
" " , 50 per cent cals.	7.3	2.4	4.5	4.5	4.9	3.5	1.4	3.9	6.1
Rat liver, AAB	5.7	2.3	4.3	4.2	4.9	3.8	1.4	3.8	5.4
" " , *m*'DAB, non-T. lobe	7.3	2.2	4.2	3.9	5.1	3.9	1.3	3.6	5.3
Rat liver, *m*'DAB, cirrhosis	6.0	2.2	3.4	4.3	4.8	3.6	1.2	2.8	4.8
II. Rat tumors									
Flexner-Jobling carc.	8.3	2.0	3.7	4.3	4.8	3.1	0.9	3.5	5.3
Sarcoma MC subcu.	7.4	2.0	3.8	5.4	5.2	5.1	1.0	3.4	4.4
Hepatoma DAB	6.5	1.9	4.4	4.7	5.6	4.5	1.0	3.6	4.9
" *m*'DAB	7.9	2.0	3.8	5.1	4.8	4.2	1.2	3.3	4.7
III. Rat spont. mammary fibrosarcoma	5.2	1.2	3.5	9.8	4.7	2.4	.26	1.7	3.2
IV. Miscellaneous									
Rabbit spont. skin T.	7.0	1.7	3.1	5.1	4.6	3.6	.57	2.9	4.3
Human T. of colon	7.7	1.4	3.4	6.4	4.3	3.2	.85	3.1	5.3
" colon tissue	8.1	2.0	3.7	5.1	4.8	3.6	1.3	3.6	5.3

TABLE 4

COMPARISON OF AMINO ACIDS IN CERTAIN ANIMAL TISSUES

(All values are percentages of amino acids in fat-free, dry material corrected to 16 per cent N)

	4 Tumors (present study)	Fibrosarcoma (4)	Normal rat tissues	Beef (6, 8, 12, 14)	Pork (6, 12, 14, 21)	Mammary fibrosarcoma	Gelatin or collagen (5)
				(per cent)			
Arginine	5.4– 6.2	5.9	5.0– 6.0	6.2	6.2	6.8	7.3–9.0
Glutamic acid	11.7–13.3	12	10.4–15	15.2		10.2	11.4
Glycine	5.1– 6.3	4.5	4.9– 6.1	5.0		17.1	24–30
Histidine	1.9– 2.7	2.5	2.0– 2.4	3.5	3.2	1.2	0.7–0.9
Isoleucine	4.3– 5.6	4.5	4.8– 5.2	5.3	4.8	2.5	1.3–1.8
Leucine	7.2– 8.9	7.5	7.3– 9.1	7.8	7.8	4.7	3.1–3.3
Lysine	6.5– 8.3	7.5	6.5– 8.3	8.0	8.1	5.2	3.2–3.7
Methionine	1.9– 2.0	1.8	2.2– 2.5	2.7	2.5	1.2	0.8–0.9
Phenylalanine	3.7– 4.4	3.8	3.4– 4.5	3.9	4.0	3.5	2.0–2.4
Threonine	3.1– 5.1	3.8	3.5– 4.1	4.5	4.6	2.4	1.7–2.4
Tryptophan	0.9– 1.0		1.2– 1.4	1.1	1.1	0.26	
Valine	4.4– 5.3	5.2	4.3– 6.1	5.2	5.2	3.2	2.4–2.8

The most obvious finding in the present study is the general similarity in the proportions of the various amino acids found in different tumors and the further similarity between these proportions and those of normal rat muscle, normal rat liver, or even of various cuts of beef, pork, or mutton. In other words, the gross amino acid composition of tumors is like that of most other animal tissues.

It has long been taken for granted that the characteristic behavior of tumors depends on a peculiar deviation from normal in chemical structure. The present results do not, however, eliminate protein from this consideration; abnormal or tumor protein may very well exist, but the abnormality is not in the gross proportions of the amino acids contained therein. Rather, it would have to be in the arrangements of the amino acids within the molecule or in more subtle aspects of protein structure. Another possibility is that the amino acid composition of a specific cellular fragment may be abnormal, and studies of cellular fractions have recently been made by Schweigert *et al.* (22).

The present results do not support the concept of Kögl and Erxleben (10) that tumors are characterized by high amounts of D-glutamic acid. Essentially similar results for glutamic acid were obtained when the assay organism was *L. lycopersici*, which responds to the DL form of the amino acids (11), as with *Leuc. mesenteroides* (24) and *S. faecalis* (3), which respond and only to the L form. In fact, the discrepancy in favor of *L. lycopersici* was greater for hydrolyzates from normal liver, 14.7 per cent versus 14.2 per cent, than for a tumor hydrolyzate, 13.5 per cent versus 13.3 per cent. Nor do the present results suggest that the arginine content of tumors is characteristically high. This latter acid was emphasized by Dunn *et al.* (4), who analyzed many samples of a transplantable fibrosarcoma and compared it with rat connective tissue after subtraction of the amino acids calculated to be in the collagen present. That this latter substance can exert a profound effect on the amino acid composition of the entire tissue is shown in the analyses of our rat mammary fibrosarcoma, which was characterized by unusually high values for glycine, proline, and arginine.

SUMMARY

1. Samples of the Flexner-Jobling carcinoma, a sarcoma due to methylcholanthrene, hepatomas due to *p*-dimethylaminoazobenzene and *m'*-methyl-*p*-dimethylaminoazobenzene, and a spontaneous mammary fibrosarcoma were analyzed microbiologically for eighteen amino acids. Normal and pre-neoplastic tissues were analyzed by similar technics.

2. All eighteen amino acids were present in all the tumors. The amounts of the amino acids in most of the tumors were found to be very similar to those in the normal rat tissues analyzed, and to those in ordinary cuts of beef or pork. The mammary fibrosarcoma proved to be exceptional in that it contained very high amounts of glycine and proline, and in general it resembled the composition of a mixture of ordinary tumor tissue diluted with connective tissue.

REFERENCES

1. Block, R. S., and Mitchell, H. H. The Correlation of the Amino-Acid Composition of Proteins with Their Nutritive Value. Nutrition Abstr. & Rev., **16**:249–78, 1946–47.
2. Brand, E. Amino Acid Composition of Simple Proteins. Ann. New York Acad. Sc., **47**:187–228, 1946.
3. Dunn, M. S.; Camien, M. N.; Shankman, S.; and Block, H. The Determination of D(−)- and L(+)-Glutamic Acid in Protein Hydrolysates. J. Biol. Chem., **168**:43–49, 1947.
4. Dunn, M. S.; Feaver, E. R.; and Murphy, E. A. The Amino Acid Composition of a Fibrosarcoma and Its Normal Homologous Tissue in the Rat. Cancer Research, **9**:306–13, 1949.
5. Graham, C. E.; Waitkoff, H. K.; and Hier, S. W. The Amino Acid Content of Some Scleroproteins. J. Biol. Chem., **177**:529–32, 1949.
6. Greenhut, I. T.; Sirny, R. J.; and Elvehjem; C. A. The Lysine, Methionine and Threonine Content of Meats. J. Nutrition, **35**:689–701, 1948.
7. Halwer, M., and Nutting, G. C. Cysteine Losses Resulting from Acid Hydrolysis of Proteins. J. Biol. Chem., **166**: 521–30, 1946.
8. Hier, S. W.; Graham, C. E.; Friedes, R.; and Klein, D. The Microbiological Determination of Amino Acids in Animal Proteins. J. Biol. Chem., **161**:705–16, 1945.
9. Hill, E. G., and Patton, A. R. The Maillar Reaction in Microbiological Assay. Science, **105**:481–82, 1947.
10. Kögl, F., and Erxleben, H. Zur Aetiologie der malignen Tumoren. I. Mitteilung über die Chemie der Tumoren. Ztschr. f. physiol. Chem., **258**:57–95, 1939.
11. Kuiken, K. A.; Lyman, C. M.; and Hale, F. Factors Which Influence the Stability of Tryptophan during the Hydrolysis of Proteins and Alkaline Solution. J. Biol. Chem., **171**:551–60, 1947.
12. Lyman, C. M., and Kuiken, K. A. The Amino Acid Composition of Meat and Some Other Foods. Texas Agr. Exp. Sta. Bull. No. 708, 1949.
13. Mohammad, A.; Fraenkel-Conrat, H.; and Olcott, H. S. The "Browning" Reaction of Proteins with Glucose. Arch. Biochem., **24**:157–78, 1949.
14. Neilands, J. B.; Sirny, R. J.; Sohljell, I.; Strong, F. M.; and Elvehjem, C. A. The Nutritive Value of Canned Foods. II. Amino Acid Content of Fish and Meat Products. J. Nutrition, **39**:187–202, 1949.
15. Olcott, H. S., and Fraenkel-Conrat, H. Formation and Loss of Cysteine during Acid Hydrolysis of Proteins. Role of Tryptophan. J. Biol. Chem., **171**:583–94, 1947.
16. Patton, A. R., and Hill, E. G. Inactivation of Nutrients by Heating with Glucose. Science, **107**:68–69, 1948.
17. Pearce, E. L.; Sauberlich, H. E.; and Baumann, C. A. Amino Acids Excreted by Mice Fed Incomplete Proteins. J. Biol. Chem., **168**:271–82, 1947.
18. Sauberlich, H. E. Metabolism of Amino Acids by Normal and Deficient Rats and Mice. Thesis, University of Wisconsin, 1948.

19. SAUBERLICH, H. E., and BAUMANN, C. A. The Effect of Dietary Protein upon Amino Acid Excretion by Rats and Mice. J. Biol. Chem., **166**:417–28, 1946.

20. ———. A Microbiological Determination of Alanine. *Ibid.*, **177**:545–51, 1949.

21. SCHWEIGERT, B. S.; GUTHNECK, B. T.; KRAYBILL, H. R.; and GREENWOOD, D. A. The Amino Acid Composition of Pork and Lamb Cuts. J. Biol. Chem., **180**:1077–83, 1949.

22. SCHWEIGERT, B. S.; GUTHNECK, B. T.; PRICE, J. M.; MILLER, J. A.; and MILLER, E. C. Amino Acid Composition of Morphological Fractions of Rat Livers and Induced Liver Tumors. Proc. Soc. Exper. Biol. & Med., **72**: 495–501, 1949.

23. SNELL, E. E. The Microbiological Assay of Amino Acids. Advances in Protein Chemistry, Vol. 2, pp. 85–118. New York: Academic Press, 1945.

24. STEELE, B. F.; SAUBERLICH, H. E.; REYNOLDS, M. S.; and BAUMANN, C. A. Media for *Leuconostoc mesenteroides* P-60 and *Leuconostoc citrovorum* 8081. J. Biol. Chem., **177**: 533–44, 1949.

25. STOKES, J. L.; GUNNESS, M.; DWYER, I. M.; and CASWELL, M. C. Microbiological Methods for the Determination of Amino Acids. II. A Uniform Assay for the Ten Essential Amino Acids. J. Biol. Chem., **160**: 35–49, 1945.

26. TOENNIES, G. Protein-Chemical Aspects of Cancer. Cancer Research, **7**:193–229, 1947.

Book Reviews

Cancer: A Manual for Practitioners. 2d Ed. Published by the American Cancer Society (Massachusetts Division), Inc. Agnet: Rumford Press, 1950.

This book contains a clear concise discussion of cancer of nearly every organ of the body. Emphasis is placed on information which will aid in the diagnosis of cancer. Treatments, which are presently considered the best, are suggested. No attempt is made to give details of surgical, radiological, or other forms of treatment. There is a section on care of the patient with advanced cancer.

Cancer of each organ is discussed by a writer well informed on the subject and in most cases by an authority. The book is thoroughly up-to-date, with the recent advances in knowledge of cancer. It fulfills its purpose as a manual on cancer and will be profitable reading as well as a handy reference for every doctor who sees patients. It has not been prepared for the oncologist or cancer control specialist who would find it filled with practical information but lacking in academic details. There are short discussions on historical trends in cancer, cancer research, epidemiological aspects of cancer, cancer control programs, and suggestions for talks on cancer to lay audiences.

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Advances in Enzymology, Vol. 10, F. F. NORD, ED. New York: Interscience Publishers, 1950. $7.50.

This volume follows to a considerable extent the pattern of those previous to it: It contains articles on enzymology in the strictest sense, on the organic chemistry of compounds whose relation to enzymology is yet unknown, and on technologically important processes whose mechanisms are an enigma. This selection undoubtedly reflects the individual preferences of the editor. Some articles in this and the preceding volumes are critical essays, embodying a considerable degree of original thought, but most are reviews of factual material.

Tage Astrup has reviewed blood clotting in much the same fashion as the subject might be covered in the *Annual Reviews of Biochemistry*, with 328 references for 39 pages of text. After reading the article, this reviewer confesses that his confusion on this subject continues unabated, and the anarchy referred to by Chargaff apparently still exists. One is entitled to ask the investigators in the field whether it is really impossible to create an understandable, comprehensive, and cohesive synthesis of the available experimental data. Certainly, it has yet to be done.

Articles on tryptophanase by F. C. Happold and on sucrose phosphorylase by W. Z. Hassid and M. Doudoroff are good accounts of these relatively simple reactions. In the latter article, the clarity of the presentation and the work it represents inspire envy and admiration.

The phosphatases now occupy the same vexing position once assumed by the peptidases. Many investigators are bound to attribute the cleavage of a wide number of substrates to a very limited number of enzymes, usually the so-called "alkaline" and "acid" phosphatases. J. Roche and N-v. Thoai, writing on "alkaline phosphatase," while citing evidence to the contrary, seem inclined to establish that there is indeed the one true phosphatase. However, their summary is informative.

C. Martius and F. Lynen set forth a discussion of the citric acid cycle, which, while of great interest, comes at an unfortunate time. The subject could probably be presented as a more complete picture a few months hence, when current researches on the Coenzyme A carriage of acetate and its relation to active acetate are more fully developed.

A comprehensive report on sulfur metabolism in plants by T. Bersin summarizes in considerable detail the various types of sulfur compounds that occur and the good studies that have been made with these somewhat neglected compounds.

The final articles on changes in harvested tobacco leaves, by W. G. Frankenburg, and on the assimilation of hydrocarbons, by C. E. Zobell, seem to be out of place in this book. The topics may or may not be of importance to the industries concerned (the reviewer is not qualified to say), and the competence of the authors is not in dispute. These articles present a collection of facts on, respectively, the gross changes in the composition of a particular plant leaf under certain empirical conditions, and observations on the over-all ability of various microörganisms to utilize some pure hydrocarbons and some hydrocarbon mixtures of very complex composition. While one can hardly chastise the authors for failing to deal in terms of reactions and mechanisms in such complicated systems, an indictment can be pressed against the editor for including such presentations as advances in enzymology and *related* topics. Exactly the same argument can be applied even to the excellent article describing the brilliant organic chemistry involved in the elucidation of the structure of streptomycin by N. G. Brink and K. Folkers. While a relation to enzymology undoubtedly exists in these various topics, no one appears to be in a position to discuss it.

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